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14. ABSTRACT
THIS WAS A 5-YEAR PROJECT TO EXPERIMENTALLY ASSESS THE NEUROTOXICOLOGIC POTENTIAL OF EXPOSURE TO DEPLETED URANIUM (DU), AND THE ROLE OF STRESS IN ALTERING THE TOXICITY. THE PROJECT INVOLVED DETERMINATION OF THE TOXICOKINETICS OF DU IN THE BRAIN, DEVELOPMENT OF A RELEVANT STRESS MODEL, AND STUDY OF NEUROTOXIC EFFFECTS OF A SINGLE (ACUTE) EXPOSURE TO SOLUBLE DU AND TO LONG-TERM EXPOSURE TO IMPLANTED PELLETED DU, AND THEIR MODIFICATION BY STRESS. MAJOR FINDINGS IN THE ACUTE STUDY WERE DOSE-RELATED ELEVATED URANIUM IN BRAIN REGIONS, AND TRANSIENT DECREASE IN DOPAMINE IN THE STRIATUM ON POST-DOSING DAY 3 IN THE HIGH DOSE UNSTRESSED ANIMALS. THERE WERE DU-RELATED DECREASES IN MOTOR ACTIVITY, BODY WEIGHT GAIN AND FORELIMB GRIP STRENGTH. TRANSIENT UREMIA FROM DU DOSE-RELATED RENAL TUBULAR NECROSIS WAS ALSO SEEN, AND MAY HAVE CONTRIBUTED TO THESE CLINICAL FINDINGS. STRESS DID NOT ENHANCE THE DU TOXICITY. MAJOR FINDINGS FROM THE LONG-TERM DU-IMPLANTATION/STRESS STUDY WERE DU DOSE-RELATED INCREASED URANIUM CONCENTRATIONS IN SERUM, KIDNEY AND BRAIN REGIONS IN RATS SACRIFICED 6 MONTHS POST-EXPOSURE, UNAFFECTED BY STRESS. DECREASE IN DOPAMINE IN THE STRIATUM AND EPINEPHRINE IN THE CEREBELLUM WERE SEEN IN THE HIGH DOSE DU GROUP, ALSO UNMODIFIED BY STRESS. THESE STUDIES SHOW THAT URANIUM MOBLIIZED FROM PERIPHERAL SITES CAN ENTER THE BRAIN AND HAVE ADVERSE NEUROLOGIC EFFECTS.

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Depleted uranium, neurotoxicity. stress

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INTRODUCTION

This is a five-year study on the neurotoxic potential of depleted uranium (DU) in laboratory rats. Previous studies with Gulf War veterans and experimental animals exposed to embedded DU suggest that neurotoxicity may result from DU exposure (McDiarmid *et al.* 2000; Pellmar *et al.*, 1999). The current investigation is designed to assess the neurotoxic potential of acute and chronic exposure to DU and the contribution of stress to expression of DU neurotoxicity and kinetics. All studies were performed with adult male Sprague-Dawley rats. As noted in the Table below, the components of this project are as follows:

1 - Dose-finding and preliminary toxicokinetic studies of DU. These were conducted in Year 1 of the project.

2 - Pilot stress study - To obtain an optimal model of experimental stress for use in the acute and chronic DU exposure studies. This was done in Years 2 and 3.

3 - Acute neurotoxicity of soluble DU (uranyl acetate) - To determine nervous system kinetics and toxicity of DU. This was done in Years 3 and 4. The study consisted of pre-dosing stress, followed by a single exposure to three levels of DU. Endpoints were in-life neurobehavior, sequential sacrifice with tissue uranium determination, neurochemistry, clinical chemistry and nervous system and renal histopathology.

4 - Long-term toxicity study - This was to assess the neurotoxic effects of chronic exposures from implanted solid DU particles, a model of some Gulf War casualties. DU dosing was done by intramuscular implantation of pellets at four levels. This was followed by multiple stress exposures and sequential neurobehavioral evaluations and plasma corticosterone determinations. Terminal sacrifice was at six months, with tissue uranium concentrations, neurochemistry, clinical chemistry and nervous system and renal histopathology being done in Years 4 and 5.

Methods for both the acute and long-term toxicity (3, 4 - above) studies employed the following procedures. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis was used to assess the kinetics of uranium in the cerebral cortex, hippocampus, striatum, and cerebellum at selected periods after DU administration. Neurotoxicity was assessed with behavioral, morphological, and biochemical endpoints. Behavioral assessment of neurotoxicity utilized the Functional Observation Battery (FOB), motor activity, and tests of learning and memory (by passive/active avoidance). Biochemical analyses included quantification of neurotransmitters (dopamine, norepinephrine, serotonin, glutamate, GABA), determination of receptor number and indicators of oxidative stress (levels of oxidized and reduced glutathione), done in the same brain regions examined for DU kinetics. Morphological studies employed perfusion-fixation, multilevel sampling of the nervous system and of the kidney, using contemporary light microscopic procedures to allow detailed evaluation of any lesions. Based upon the above noted pilot stress study, a model of variable daily restraint, handling or swimming stress was employed, in association with DU exposure for the chronic study. These studies helped define the neurotoxic potential of DU and assess the role of stress in modifying this effect.

This is the final report for what was originally a four-year study, but which was extended to five years (ending November 30, 2006). In addition to containing a summary of the five years, this report contains detailed findings that were not reported in previous annual reports. The table that follows cites the location of study data by Annual and Final Reports.

Presentation of Data by Report

* not evaluated
◖ = partial data presentation
● = complete data presentation

		Annual Report				Final Report
Task	***Evaluation***	***Year 1***	***Year 2***	***Year 3***	***Year 4***	
1 Preliminary Studies	Dose Range Finding	●				
2 DU Kinetic Study	Serum uranium concentrations	◖	●			
	Regional brain uranium concentrations	◖	●			
	Stress effects	◖	●			
3 Pilot Stress Study	Acute stress model validation		●			
	Chronic stress model validation		●			
4 Acute Toxicity Study	**Behavioral**					
	FOB			●		
	Motor Activity			●		
	Passive-Avoidance					
	Uranium concentration (icp-ms)					
	Regional brain			◖	●	
	Blood serum			◖	●	
	Kidney				●	
	Neurochemistry					
	Neurotransmitters: dopamine, serotonin, epinephrine			●		
	Neurotransmitters: GABA			●		
	Neurotransmitters: glutamate			*		
	Reduced glutathione			◖	●	
	Oxidized glutathione					●
	Neurotransmitter receptor numbers				◖	●
	Pathology					
	Nervous tissue: H&E			●		
	Nervous tissue: T. blue					●
	Nervous tissue: GFAP					●
	Nervous tissue: F-Jade					●
	Kidney			◖	●	
	Clinical chemistry			◖	●	
	Stress model: CORT determinations			●		

		Annual Report				Final Report
Task	***Evaluation***	***Year 1***	***Year 2***	***Year 3***	***Year 4***	
5 Chronic Toxicity Study	**Behavioral**					
	FOB				●	
	Motor Activity				●	
	Passive-Avoidance				●	
	Active Avoidance				●	
	Uranium concentration (icp-ms)					
	Regional brain				●	
	Blood serum				●	
	kidney				●	
	urine				●	
	Neurochemistry					
	Neurotransmitters: dopamine, serotonin, epinephrine				●	
	Neurotransmitters: GABA					●
	Reduced/oxidized glutathione				◖	●
	Neurotransmitters: glutamate					●
	Neurotransmitter receptor numbers					●
	Pathology					
	Nervous tissue: H&E					●
	Nervous tissue: T. blue					●
	Nervous tissue: GFAP					●
	Nervous tissue: F-Jade					●
	Kidney				●	
	Clinical chemistry					●
	Thymus/adrenal weights				●	
	Stress model: CORT determinations				●	

BODY

I. Tasks from Statement of Work (as revised 1/15/05) and Accomplishments- This was a complex, five-year study on the neurotoxic potential of depleted uranium and its modulation by stress. What follows are tasks (italics, underlined) described in the approved Revised Statement of Work (see Appendix) along with relevant research accomplishments and references to previously reported data (italics, bolded).

A. Task 1 - *Perform preliminary studies to identify appropriate doses of DU for kinetic studies.*

Research Accomplishments for Task 1. Preliminary studies were conducted in Year 1 to determine doses of uranyl acetate to be used in kinetic and toxicity studies. ***Data was presented in the Year 1 (10/2002) Annual Report.*** Based upon these studies, single exposures of 1 and 10 mg/kg of uranium (given intraperitoneally as uranyl acetate) were chosen for the kinetic studies.

B. Task 2 - *Perform a toxicokinetic study to determine the concentration of DU in rat serum, cerebral cortex, hippocampus, striatum, and cerebellum at several times after DU exposure. The effect of stress on DU kinetics will also be determined.*

Research Accomplishments for Task 2. The in-life portion of the toxicokinetic studies were completed in Year 1 and data analysis was completed in Year 2. ***The results of the studies were presented at the Force Health Protection Review in October 2002 and the complete data was presented as an addendum to the Year 2 (10/2003) Annual Report. A paper reporting these results has been published- D.S. Barber, M.F. Ehrich and B.S. Jortner, The effect of stress on the temporal and regional distribution of uranium in rat brain after acute uranyl acetate exposure. Journal of Toxicology and Environmental Health, Part A 68, 99-111, 2005 (included in Year 3 [10/2004] Annual Report).***

The results of the toxicokinetic study demonstrated that a single peripheral exposure to soluble depleted uranium increased central nervous system concentrations in certain brain regions. Uranium appears to enter the brain rapidly, and peak uranium concentrations occur within 8 hours in most regions. Of the latter, the hippocampus and striatum contained the highest levels of uranium after 8 hours of exposure in both stressed and unstressed rats. More than one phase of uranium clearance was observed, with the terminal phase being much slower than the initial one. Uranium levels in hippocampus, cerebellum and cortex remained elevated 7 days post-exposure. Stress exposure significantly reduced uranium

levels in hippocampus and cerebellum 24 hours after administration and tended to reduce uranium concentrations in all brain regions at 7 days.

C. ***Task 3***- *Perform a pilot stress study, to determine the most appropriate model of stress to employ in subsequent acute and long-term DU neurotoxicity studies.*

Research Accomplishments for Task 3. The pilot study was completed in Year 2. ***The results of the study were reported in the Annual Reports for Years 2 (10/2003) and 3 (10/2004). A paper reporting the results is in press, S. Hancock, M. Ehrich, J. Hinckley, T. Pung, B.S. Jortner. The effect of stress on the acute neurotoxicity of the organophosphate insecticide chlorpyrifos. Toxicol. Appl. Pharmacol. in press (see Appendix).***
The study was conducted with two objectives 1) to determine the effect of daily (5 days/week) exposure to various stress regimens (routine handling, restraint, forced swimming and a combination of restraint/forced swimming) on plasma corticosterone levels and 2) whether the effect would continue over a prolonged time period. The major findings were that, of the stressors evaluated, only forced swimming, alone or in combination with restraint, produced a significant (but transient) elevation of plasma corticosterone levels and the effect continued for up to 7 weeks. The results also showed that animals habituated to the restraint stress quite quickly and there was no significant difference between the plasma corticosterone levels of animals stressed by restraint compared to the control. These results were used in the design of the long-term study described below (Task 5). The animals exposed to forced swimming with or without restraint had atrophy of basilar dendrites of CA1 hippocampal pyramidal neurons as demonstrated by Golgi staining (see Activities Outside Statement of Work). ***Results were reported in Year 3 (10/2004) Annual Report.***

D. ***Task 4***- *Perform an acute toxicity study evaluating the effects of several doses of soluble DU and of stress, as measured by neurobehavioral, neurochemical and neuropathological endpoints and regional brain uranium concentrations.*

Research Accomplishments for Task 4. The in-life part of the acute toxicity study was completed in Year 3. ***The results of the behavioral, neurochemical and uranium concentration data were reported in the Annual Reports of Years 3 (10/2004) and 4 (10/2005). These are also included in two manuscripts in the Appendix (Barber et al., Neurologic effects of acute uranium exposure with and without exposure [for submission to Toxicological Sciences]; Zimmerman et al., Temporal clinical chemistry and microscopic renal effects following acute uranyl acetate exposure [for submission to***

Toxicologic Pathology]). Summary of findings for the DU acute toxicity study, including neuropathological data not previously reported, is as follows.

1. Study Design- The study was conducted in five experimental blocks to provide an n=5/treatment group/terminal endpoint as shown in Figure 1.
   a. DU dosing- The approach featured the use of single intramuscular doses of 0.1, 0.3 and 1.0 mg/kg of soluble DU (uranyl acetate) in male Sprague-Dawley rats. There was one week predosing administration of stress consisting of four consecutive days of restraint stress followed by one day of forced swimming.
   b. In-life measurements- At the cessation of swim stress, animals were bled for subsequent corticosterone determination and immediately dosed intramuscularly with DU. The Functional Observational Battery, motor activity assessment and passive-avoidance testing were performed weekly.
   c. Terminal measurements- Cohorts of rats were sacrificed on post-dosing days 1, 3, 7 and 30 for tissue uranium concentration, neurochemistry or pathology. See Table 1.

Table 1. In-life evaluations and final disposition of experimental animals by post-dosing day for the acute toxicity study.

	In-Life and Terminal Endpoints				
Post-Dosing Day	**Behavior** (FOB, motor activity, passive avoidance)	**Neurochemistry** (regional brain transmitters, receptor levels and oxidative stress, kidney pathology, clinical chemistry	**Uranium concentration** (regional brain, kidney and serum uranium, renal pathology)	**Pathology** (CNS, PNS)	**Total *n*/group**
1	*n*=0	*n*=5/group	*n*=5/group	*n*=0	*n*=10/group
3	*n*=0	*n*=5/group	*n*=5/group*	*n*=5/group	*n*=10-15/group*
7	*n*=0	*n*=5/group	*n*=5/group*	*n*=5/group	*n*=10-15/group*
30	*n*=10-15/group**	*n*=5/group	*n*=5/group*	*n*=5/group	*n*=10-15/group*

*Days 3, 7, 30: no 0.1 mg/kg uranium rats dosed for uranium concentration determinations
**Behavioral determinations were performed on all rats assigned to the 30 day sacrifice

2. Corticosterone Levels at Dosing- There was a four-fold elevation of plasma corticosterone levels in stressed rats due to forced swimming immediately prior to dosing.
3. Neurobehavioral Findings (Note: Day 0 = 1st day of stress, numbers in brackets refer to DU post-dosing day)- Uranium exposure led to significant decreased forelimb grip strength (dose-related), ambulatory movements and body weight gain. Pre-dosing stress had no effect on these measurements. There was a

significant decrease in performance on the passive avoidance test for all animals over time which is expected following a single training period (Tables 1A-3A, Appendix). Unstressed animals treated with 1.0 mg uranium/kg performed similarly to controls on day 12 [8 days post-dosing] but exhibited significantly poorer performance (decreased latency) on day 19 [15 days post-dosing] ($p=0.04$). These animals also tended to have poorer performance on day 26 [post-dosing day 22]. Latency time returned to control levels by day 31 [27 days post-dosing] (Figure 1). A similar trend was observed in animals treated with 0.3 mg uranium/kg. Animals that were stressed prior to uranium exposure exhibited the same performance as controls at all timepoints, indicating that prior stress prevented the adverse effects of uranium on memory.

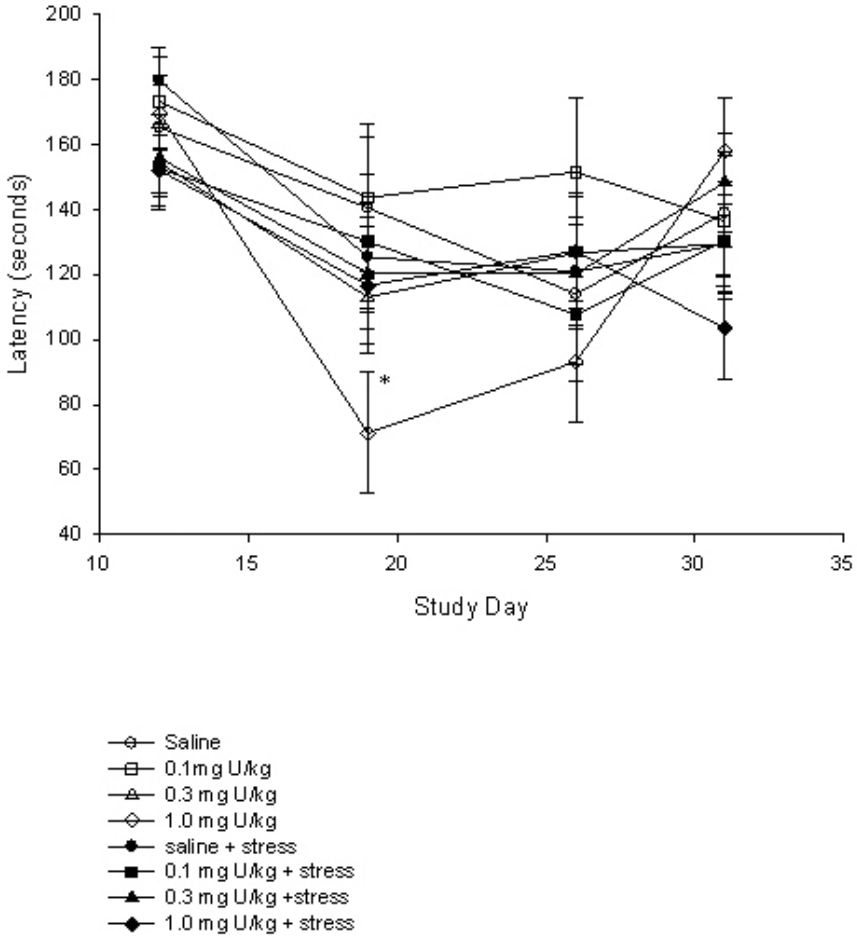

Figure 1. Passive Avoidance Latency - Acute Toxicity Study. Values are mean ± SEM (N=10-15 observations). * indicates values that are significantly different from saline only ($p<0.05$). Day 0 is the 1st day of exposure to stress. Animals were dosed with DU on Day 4.

4. Tissue and Serum Uranium Concentrations- Exposure to uranium resulted in a dose-dependent increase in uranium in serum and brain cortex, cerebellum, hippocampus and striatum. The increases were independent of stress. Unlike the brain regions, uranium levels in serum diminished after 7 days post-dosing. Peak uranium levels occurred later than observed in the toxicokinetic study (I-B above), which likely reflects slower absorption of uranium from intramuscular injection than intraperitoneal injection.

   Kidney uranium concentrations were considerably higher than in the brain. Uranium concentrations peaked on post-dosing Day 1 and progressively declined. By Day 30, uranium was only detected in the high (1.0 mg/kg) DU exposed rats, and these approached control values.

5. Neurochemistry Determinations
   a. Neurotransmitters. Tissue concentrations of transmitters (GABA, dopamine, serotonin and norepinephrine) were determined in cortex, cerebellum, striatum, and hippocampus. The concentration of dopamine in the striatum was decreased by 40% in the high dose DU group on post-dosing Day 3. This effect on dopamine was ameliorated by pre-dosing stress.
   b. Neurotransmitter Receptor Numbers. Although changes were seen in striatal dopamine levels, there was no effect of stress and/or DU on numbers of dopamine D2 receptors in the striatum. No significant changes were present in the number of NMDA receptors in hippocampus or nicotinic acetylcholine receptors in cerebral cortex at any time after uranium and/or stress exposure (Figures 2 and 3).

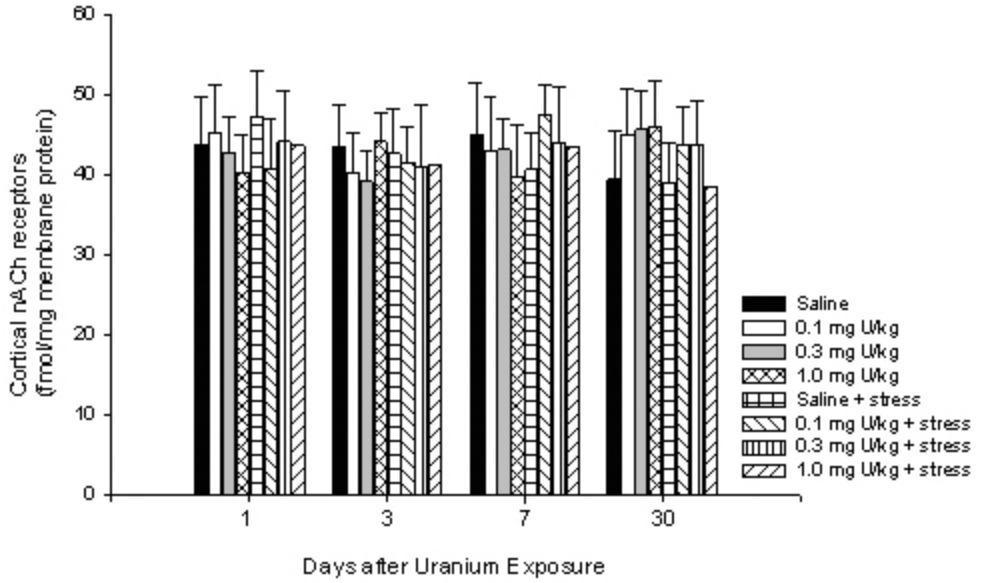

Figure 2. Acute DU Toxicity Study. Nicotinic acetylcholine receptors in cerebral cortex of rats at various times after uranium exposure with and without prior stress. Values are mean ± SD (N=4-5). There are no significant treatment or day effects on nACh receptor numbers in cerebral cortex.

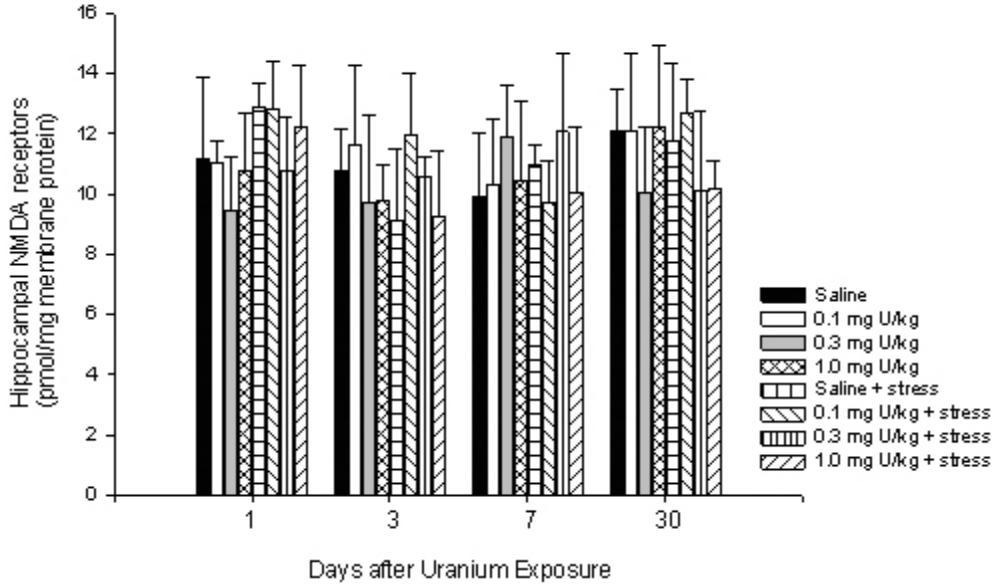

Figure 3. Acute DU Toxicity Study. NMDA glutamate receptors in hippocampus of rats at various times after uranium exposure with and without prior stress. Values are mean ± SD (N=4-5). There are no significant treatment or day effects on NMDA receptor numbers in hippocampus.

c. Glutathione assays. The amounts of reduced (GSH) and oxidized (GSSG) glutathione are commonly used measures of cellular redox state. Decreases in GSH or increases in GSSG are indicative of a more oxidizing environment within the cell. Although differences in GSH measurements were seen across brain regions (cortex, cerebellum, striatum and hippocampus) and sacrifice day, no treatment effect was detected.

6. Pathology
   a. Neuropathology- The brain was studied by light microscopy in cross-sections at levels containing the main mass of the striatum, rostral thalamus, caudal thalamus, midbrain and cerebellum/pons-medulla. Hematoxylin and eosin staining of sections from paraffin-embedded brain was used for all animals. Glial fibrillary acidic protein (GFAP) immunostaining was also used for rats from Groups 1 (negative control), 4 (high dose DU), 5 (stress control), 8 (stress plus high dose DU) sacrificed on days three and seven. The Fluoro-Jade stain for neuronal degeneration (Schmeud and Hopkins, 2000) was employed on brains from selected rats from these groups sacrificed on day three. One micron thick sections of epoxy resin embedded spinal cord, caudal medulla, optic nerve, dorsal root ganglion and multiple peripheral nerves were stained with toluidine blue and safranin, and examined by light microscopy. No lesions of the nervous system were found in any of these preparations, regardless of the presence or absence of stress and/or DU exposure. Given the neurochemical findings, components of dopaminergic pathways, such as the substantia nigra and the striatum were given special attention. As noted above, there were no histologic alterations.
   b. Renal pathology- There was uranium dose-related necrosis of renal proximal tubular epithelium with associated elevated levels of serum creatinine and blood urea nitrogen. Necrosis and elevated biochemical indicators were prominent at post-dosing days 3 and 7. Tubular regeneration also was active at day 7 and virtually complete by day 30, along with a return of serum creatinine and blood urea nitrogen to normal for all uranium exposure groups. High dose animals had residual regions of chronic interstitial nephritis and cortical scarring on day 30. Stress had no effect on the nephrotoxicity.

7. Summary of Major Findings and Interpretation from the Acute Toxicity Study- The major effects seen in this study of intramuscular exposure to a three doses of a soluble form of

depleted uranium (uranyl acetate) and pre-dosing stress to male Sprague-Dawley rats were as follows. DU exposure–related diminished grip strength, decreased ambulatory activity and diminished weight gain were noted. DU treatment also produced transient deficits in passive avoidance which is a measure of working memory. Application of stress prior to uranium exposure prevented the effect of uranium on passive avoidance. In addition, DU dose-related increases in regional brain uranium concentrations were seen. The increases were independent of stress, and diminished with time post-dosing. There were no associated lesions in brain, spinal cord or peripheral nerve and ganglia. However, the concentration of dopamine in the striatum (caudate-putamen) was decreased in the high dose DU group on post-dosing day 3. Pre-dosing stress ameliorated this effect. There was no associated change in dopamine D2 receptors in this region. Similarly, there were no changes in NMDA or nicotinic acetylcholine receptors in the cerebral cortex. While there have not been other studies on the effect of uranium on receptor number, there is evidence that exposure to lead, a chemically similar element, leads to changes in NMDA receptor number in hippocampus (Guilarte and McGlothan, 1998). However, these effects were only seen following developmental exposure and similar effects were not observed following adult exposure (Guilarte and Miceli, 1992). Long-term exposure to relatively high levels of lead has been shown to produce changes in MK-801 binding in other brain regions, including cerebral cortex, dorsal striatum and nucleus accumbens (Cory-Slechta et al., 1997). Increases in DU dose-related renal uranium concentration and associated renal tubular necrosis and elevations of serum BUN and creatinine were prominent. These uranium concentrations peaked at 1 day post-dosing, subsequently diminished, and the necrosis and elevated serum proteins were noted at 3-7 days post-dosing. Regeneration of tubular epithelium was prominent from day 7, and largely complete on day 30. Prior stress had no effect on uranium-induced renal toxicity.

The results of this study demonstrate that a single exposure to soluble DU (uranyl acetate) at doses used in this study can have adverse neurological effects. This includes transient high DU dose-associated reduction in dopamine levels in the striatum, and alteration in some neurobehavioral activities. In no case were these effects exacerbated by prior stressful stimuli and in at least some instances (transient striatal dopamine decrease, deficits in passive avoidance performance) the effects were prevented by prior exposure to stress. Reduced glutathione levels were not altered by DU and/or stress exposures, suggesting that alteration of

cellular oxidizing environment in the brain was not a factor in the above noted effects. In addition, there were no detected lesions in the nervous system (including substantia nigra and striatum). The DU exposure-related clinical effects, including diminished grip strength, motor activity and body weight gain, may have been secondary to the DU-induced kidney injury and transient uremia. The latter was observed at all uranium doses (in a dose-related fashion), and peaked at times when the clinical effects were most evident.

E. Task 5 - *Perform a long-term (chronic) toxicity study evaluating the effects of several doses of implanted DU pellets and continuous stress, as measured by neurobehavioral, neurochemical and neuropathological endpoints and regional brain uranium concentrations.*

Research Accomplishments for Task 5. This task was modified from the original Statement of Work, based upon review of the Year 1 results at the December 2002 Force Health Protection Review. Specifically, the study was changed to provide periodic peaks of elevated plasma corticosterone during the entire six-month study by inducing stress 5 days/week with a routine stressor (restraint) and a superimposed periodic novel stress (swimming) as a model of militarily relevant stress. ***The results of neurobehavioral evaluations, regional brain, serum and kidney uranium concentrations and partial neurochemical transmitter determinations were presented in the Annual Report for Year 4 (10/2004)*** and are included here for background data.

1. Study Design- The study was conducted in five experimental blocks to provide an n=5/treatment group/terminal endpoint as shown in Figure 1.
    a. DU dosing- There were 8 treatment groups (2 levels of stress, 4 levels of DU). Dosing consisted of implantation of tantalum pellets (control), DU pellets or a combination in the gastrocnemius muscles of each rat (10 pellets/leg).
    b. Stress- As a result of the pilot study, a variable, unexpected stress model was used for the chronic study. This model used a weekly random pattern of 3 days of restraint, 1 day of swimming and 1 day of handling, with the anticipation that the variable pattern might produce elevated plasma corticosterone from stress treatments other than swimming.
    c. In-life measurements- To monitor the effects of stress treatment, blood was collected periodically throughout the study period to determine plasma corticosterone levels. The Functional Observational Battery and motor activity assessments were performed in Weeks 1, 4, 8, 11, 14, 17, 20 and 23 (n=10/group). To test learning and memory, passive and active avoidance testing was performed in the last 2 months of the study.

d. Terminal measurements- At the end of 23 weeks, rats were sacrificed for tissue uranium concentration (n=5/group), neurochemistry (n=5/group) or pathology (n=5/group), as described in Table 1 for the acute toxicity study.

2. Corticosterone Levels - The stress model successfully provided a periodic elevation of plasma corticosterone levels across the six months of the study. Corticosterone was consistently increased significantly by swimming stress, but not by the other stressors. Adrenal and thymus organs were collected at sacrifice to provide an additional assessment of the stress model. There was no effect of stress on adrenal weight, but there was a trend, although not statistically significant, for a reduced thymus weight in stressed groups.
3. Neurobehavioral Findings – Administration of DU did not result in changes in any measured motor activity. There was an increase in fine motor movement of stressed animals beginning in Week 4, which led to an associated increase in total motor activity. In addition, stressed animals had increased ambulatory movement at the end of the study (Week 23). Beginning in Week 4 there was a stress-associated significant increase in landing foot splay. In addition, DU exposure resulted in a decrease in foot splay, although this was not statistically significant. Body weight gain in stressed rats decreased over the course of the study, beginning in Week 4. Although a significant effect of DU on body weight was not seen, there was a trend for the high dose DU animals to be lower than controls. No other treatment related effects were determined by the FOB. There was no effect of DU exposure or stress on measurements of passive or active avoidance.
4. Tissue Uranium Concentration - Animals exposed to DU showed a dose-dependent accumulation of uranium in serum, brain regions (cerebral cortex, striatum, hippocampus and cerebellum) and kidney. Urine concentrations of uranium reflected a trend toward a dose-related distribution.
5. Neurochemistry Determinations
   a. Neurotransmitters. Exposure to the high dose of DU elicited an approximate 15% decrease in dopamine concentration in the striatum (data provided in year 4 [10/2004] Annual Report). This finding was of interest, because a transient decrease (40%) was also seen in striatal dopamine in the acute toxicity study. Unlike the acute study, multiple exposures to stress over a 23-week period had no effect on the change in striatal dopamine. As noted above, chronic exposure to the highest dose of DU pellets (20 pellets), did produce small, but significant, reductions in striatal dopamine. Because previous work suggests that uranium exposure can alter dopamine (Bussy et al., 2006), we

examined catecholamine metabolites in the striatum. With increased turnover of dopamine, it is possible that we would observe an increase in the levels of dopamine metabolites. There was no change in striatal levels of DOPAC or homovanillic acid (HVA), which are major metabolites of dopamine (Figure 4). This suggests that the decrease in dopamine is not due to increased metabolism of dopamine but must be caused by other mechanisms which remain to be elucidated. High dose DU exposure for six months also resulted in a decrease in cerebellar norepinephrine and an almost significant decrease in cerebral cortical serotonin (data provided in year 4 [10/2004] Annual Report). No changes in levels of GABA or glutamate were observed in cortex, hippocampus, striatum or cerebellum following 6 months exposure to implanted uranium (Figures 5 and 6).

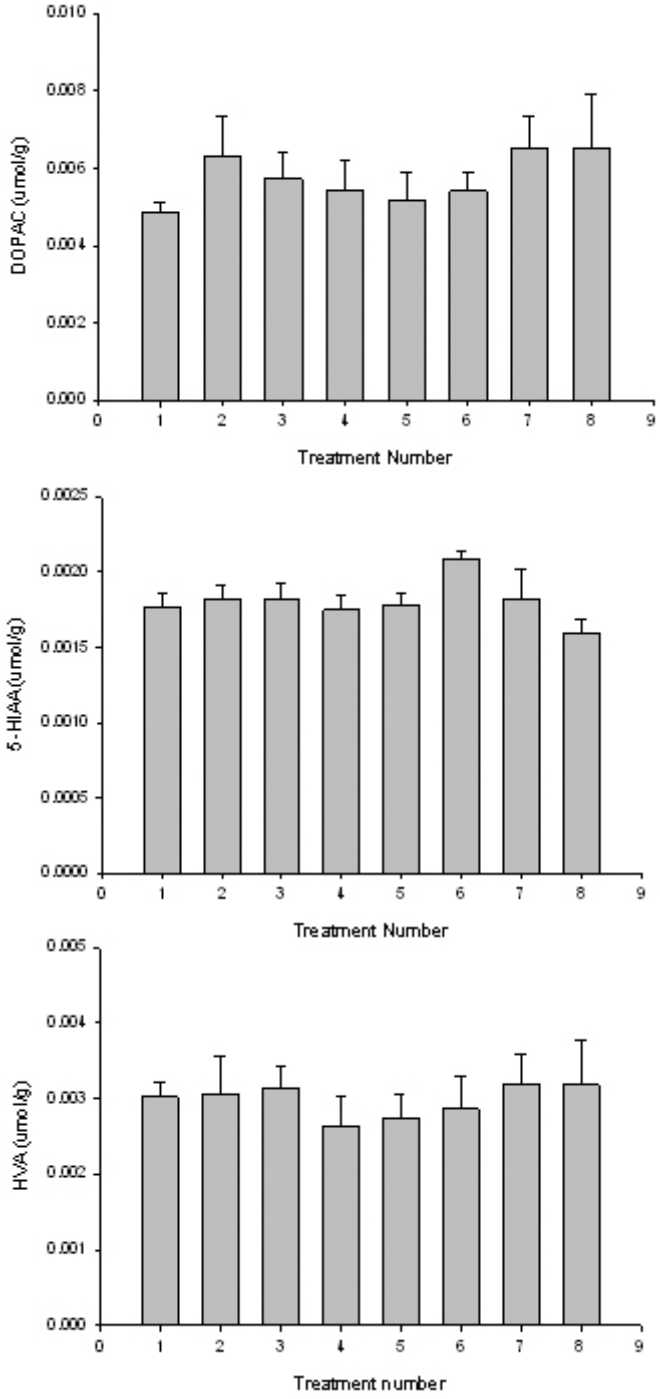

Figure 4. Long-Term DU Toxicity Study. Striatal catecholamine metabolites as a function of treatment. DOPAC and HVA are metabolites of dopamine while 5-HIAA is a serotonin metabolite. No significant effect of treatment was present on any of the above metabolites. Treatments- 1 – Tantalum control, 2 – low DU, 3 – medium DU, 4 – high DU, 5 – Tantalum + stress, 6 – low DU + stress, 7 – medium DU + stress, 8 – high DU + stress.

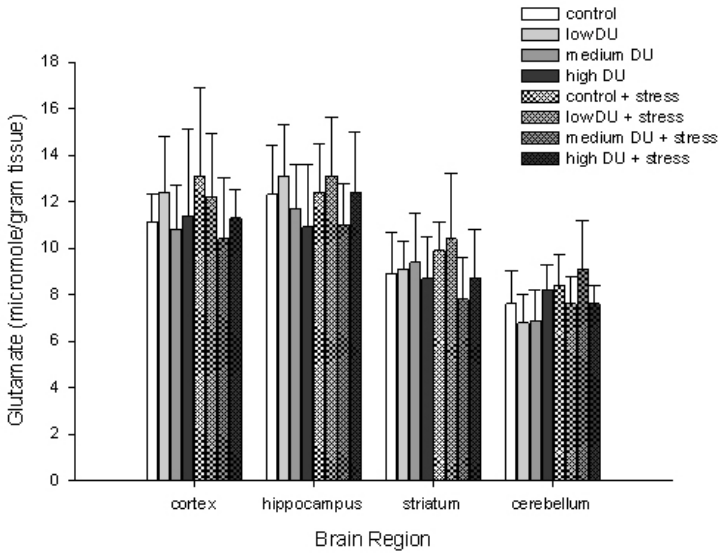

Figure 5. Long-Term DU Toxicity Study. Glutamate in various brain regions following six month exposure to implanted pellets with or without stress. Controls were implanted with tantalum pellets. Values are mean ± SD (N=4-5). No significant effect of treatment was present within a tissue but values across all treatments were significant between tissues. Hippocampus was significantly higher than cerebellum ($p<0.05$).

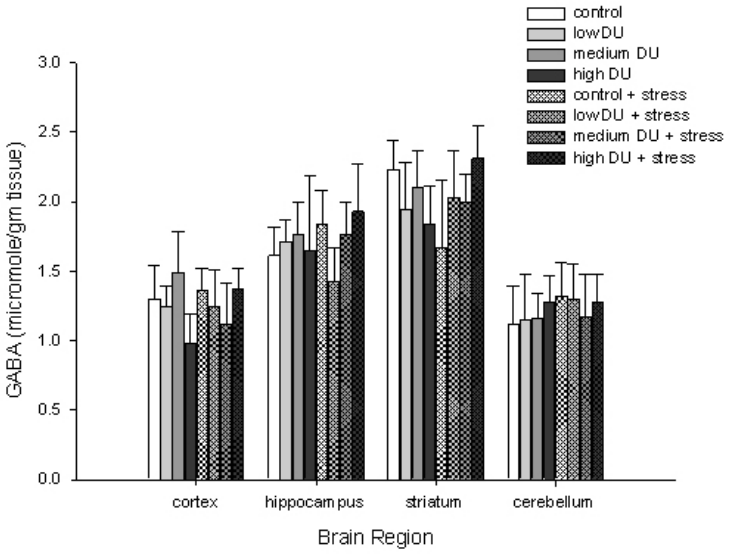

Figure 6. Long-Term DU Toxicity Study. GABA concentrations in various brain regions following six month exposure to implanted pellets with or without stress. Controls were implanted with tantalum pellets. Values are mean ± SD (N=4-5). No significant effect of treatment was present within a tissue but values across all treatments were significant between tissues. Striatum and hippocampus were significantly higher than cerebellum ($p<0.05$).

b. Neurotransmitter Receptor Numbers. Chronic exposure to implanted uranium did not alter the number of striatal D2 dopamine receptors, hippocampal NMDA receptors, or cortical nicotinic acetylcholine receptors (Figures 7, 8, 9).

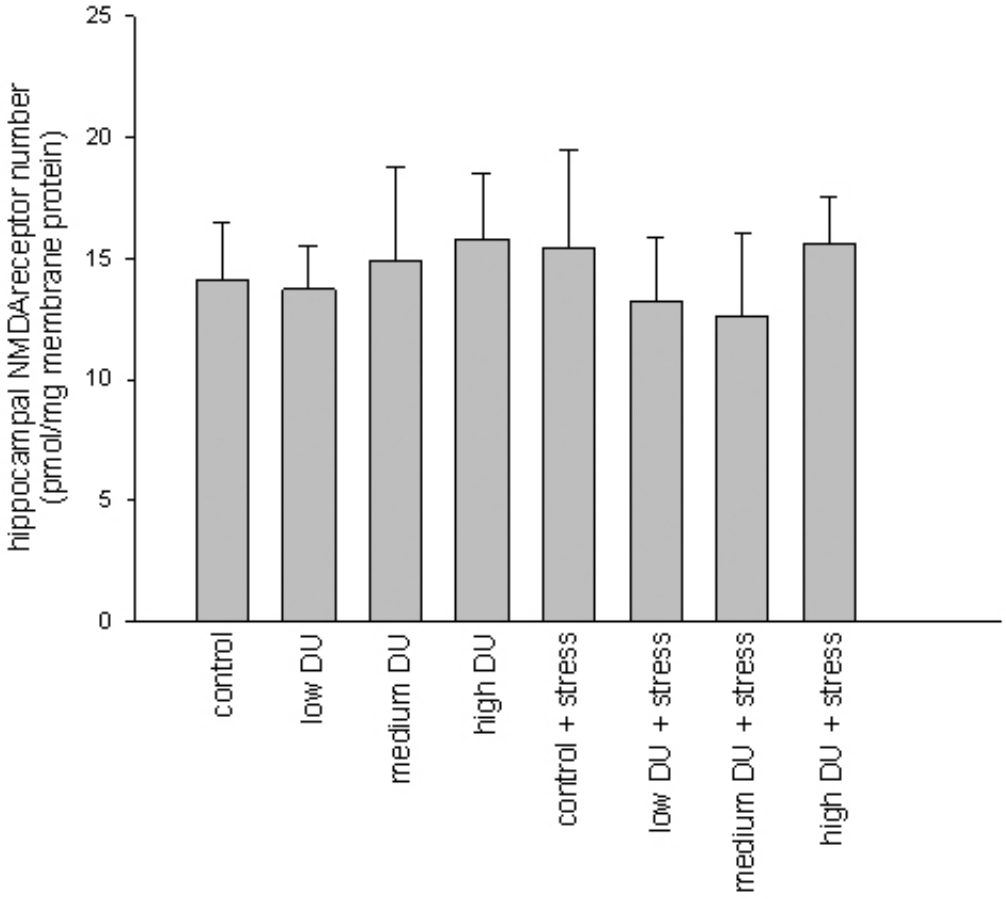

Figure 7. Long-Term DU Toxicity Study. NMDA receptors in hippocampus of rats treated for 6 months with implanted pellets. Controls were implanted with tantalum pellets. Values are mean ± SD (N=4-5). No significant differences were present among treatment groups.

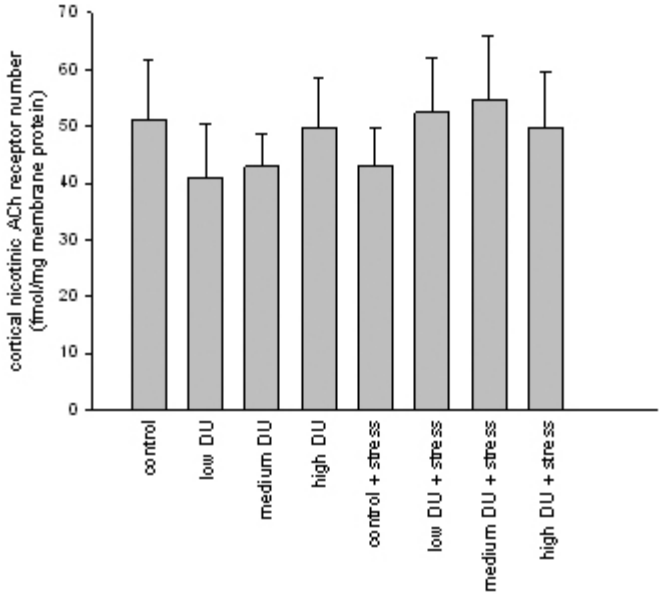

Figure 8. Long-Term DU Toxicity Study. Nicotinic acetylcholine (ACh) receptors in cerebral cortex of rats treated for 6 months with implanted pellets. Controls were implanted with tantalum pellets. Values are mean ± SD (N=5). No significant differences were present among treatment groups.

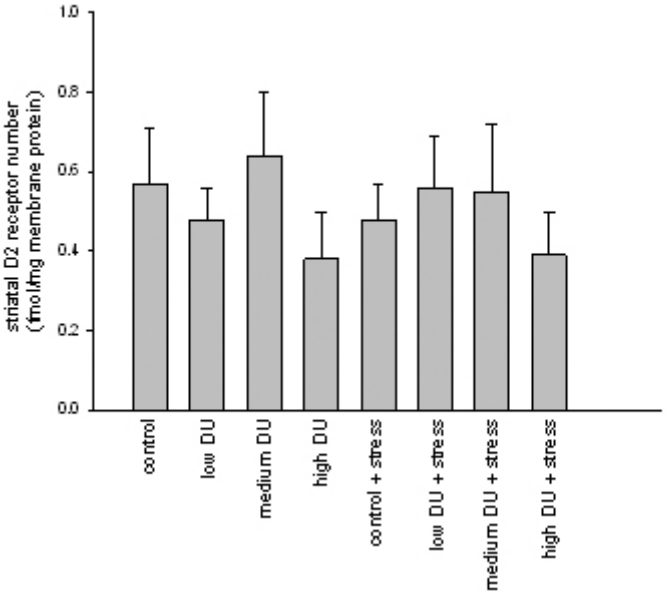

Figure 9. Long-Term DU Toxicity Study. Dopamine D2 receptors in striatum of rats treated for 6 months with implanted pellets. Controls were implanted with tantalum pellets. Values are mean ± SD (N=5). No significant differences were present among treatment groups.

c. Glutathione assays. Although reduced glutathione levels did not reveal a stress or DU treatment effect, differences in brain regions (cortex, cerebellum, striatum and hippocampus) were seen (data provided in year 4 [10/2004] Annual Report). Exposure to implanted uranium pellets for 6 months with or without concurrent stress did not produce significant effects on total glutathione concentrations in any brain region (Figure 10). As with reduced glutathione, regional differences in total glutathione were noted in the brain (Figure 10).

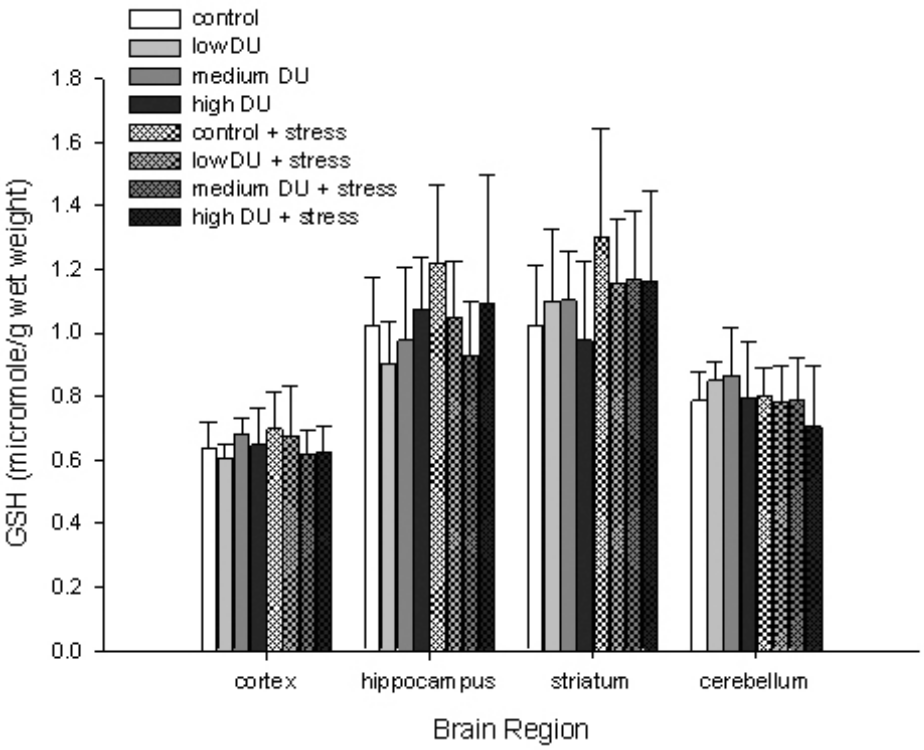

Figure 10. Long-Term DU Toxicity Study. Total glutathione in various brain regions following six month exposure to implanted uranium and/or tantalum pellets in the presence and absence of stress. Values are mean ± SD (N=5). No significant effect of treatment within a brain region are present. There are significant differences between brain regions; hippocampus and striatum have significantly higher levels of glutathione than cortex and cerebellum across all treatments ($p<0.05$).

6. **Pathology**

a. Neuropathology- Neuropathological study was extensive, and was consistent with that done in the acute DU neurotoxicity study (Task 4). This consisted of light microscopic evaluation of multiple levels of cross-sectioned

brain stained by the H&E and GFAP procedures. These sections were also evaluated using the Fluoro-Jade B fluorescent stain for degenerating neurons. One micrometer thick, toluidine blue and safranin-stained cross-sections of the medulla, cervical, thoracic and lumbar levels of spinal cord, sciatic, tibial, sural and vagus nerve, and longitudinal sections of dorsal root ganglia and adjacent spinal nerve roots were also studied by light microscopy. The GFAP, Fluoro-Jade and toluidine blue and safranin procedures were only done in animals receiving the high dose of DU with and without stress (Groups 4 and 8), stress plus tantalum (Group 5) and tantalum alone (Group 1). None of these preparations revealed DU dose related lesions in stressed or unstressed animals. There were scattered findings such as focal cavitary necrosis of the optic chiasm seen in one each rats from Group 5 (stress plus tantalum), Group 6 (stress plus low dose of DU), and Group 8 (stress plus high dose DU). It should be noted that this region was not included in the brain cross-sections of all animals. One rat in Group 5 had focal vacuolar degeneration of the spinal cord gray matter. One animal in Group 8 had early peripheral neuropathy manifest by endoneurial edema and mild axonopathy progressing to fiber degeneration. These were considered to be background changes, not related to exposure to DU and/or stress. It was concluded that such exposure to these agents, alone or in combination, did not elicit detected lesions in the central or peripheral regions of the nervous system.

Dr. Ron Mervis, Neurostructural Resaearch Laboratories, Tampa, FL, performed studies of basilar dendrites of the hippocampal CA1 pyramidal neurons using Sholl analysis of rapid Golgi method. He studied stained neurons of hippocampi from five each tantalum control and high dose DU exposed rats, with and without stress (Flood, 1993; Morest, 1981). Data indicated there was sculpting of the dendritic tree by DU and stress. Specifically, DU alone increased dendritic material in the inner 1/2 of the arbor and the combination of stress and DU elicited enhanced dendritic material in outer regions. These have been presented at the 2006 meetings of the Society of Toxicology and the Society for Neuroscience (see abstract in Appendix).

b. Renal pathology and clinical pathology- Kidney uranium concentrations in the high dose DU rats at six months were equivalent to those seen in the low dose rats of the acute toxicity study one day after intramuscular administration of

0.1 mg/kg soluble DU. However, the renal tubular epithelial necrosis seen in the acute toxicity study was not present in the high dose DU-exposed animals, despite similar concentrations of renal uranium. This is likely a reflection of the rapidity by which uranium accumulates in the kidney following exposure to soluble DU as compared to the implanted pellets. Except for one group 5 rat (stress, DU dose 0.0/mg/kg), individual and group mean serum creatinine and blood urea nitrogen values were within published reference intervals (Quimby FW, 1999) for all dosage groups. The increased serum blood urea nitrogen and creatinine results for the single group 5 rat is interpreted as being due to decreased renal perfusion secondary to preexisting renal disease or other factor (dehydration) external to the experiment. Results from the remaining group 5 rats and other study group rats, indicate that there is no DU dose, stress, or dose-stress interaction relationship with serum BUN and creatinine values. The lack of renal tubular necrosis in the high dose DU groups is reflected by the absence of changes in serum blood urea nitrogen and creatinine levels that were seen in the acute toxicity study. Histopathological examination of kidney sections from both DU exposed rats and tantalum controls did reveal the presence of a mild to minimal background nephropathy common in older animals (Percy and Barthold, 1993), of equal severity in both groups.

7. **Summary of Major Findings from the Chronic Toxicity Study -** Long-Term DU Toxicity Study (Task 5) - This was a large, complex project, involving exposure to tantalum controls and three levels of implanted DU pellets, with and without stress. The latter was applied at intervals throughout the six-month duration of the study, and was effective, based upon serially elevated plasma corticosterone levels, related to swim stress. Other stress-induced changes were body weight gain reduction and increases in ambulatory and non-ambulatory motor activity and landing foot splay. There were DU dose-related increased uranium concentrations in serum, kidney and brain regions in rats sacrificed six months post-exposure, unaffected by stress.

   Neurochemical alterations provided the most interesting findings. Significant decrease in dopamine in the striatum and epinephrine in the cerebellum were seen in the high DU dose group sacrificed at 6 months, unmodified by stress. The dopamine effect parallels a transient finding in the Acute Study (see above). Because previous work suggests that uranium exposure can alter dopamine turnover (Bussy *et al.*, 2006), we examined catecholamine metabolites in

striatum. With increased turnover of dopamine, it is possible that we would observe an increase in the levels of dopamine metabolites. However, there was no change in striatal levels of DOPAC or homovanillic acid (HVA), which are major metabolites of dopamine. This suggests that the decrease in dopamine is not due to increased metabolism of dopamine but must be caused by other mechanisms which remain to be elucidated.

Exposure to implanted uranium pellets for 6 months with or without concurrent stress did not produce significant effects on reduced or total glutathione GABA or glutamate concentrations in any brain region. This suggests that long term exposures to low levels of uranium do not cause oxidative injury to the regions of the brain that were examined. This is consistent with our findings in the acute study which also showed no effect of uranium on brain glutathione. It is also consistent with a lack of histopathological evidence of neuronal or glial injury following exposure to uranium, which would be expected in tissues with ongoing oxidative injury.

No changes in levels of GABA or glutamate were observed in cortex, hippocampus, striatum or cerebellum following 6 months exposure to implanted uranium. Previous work has demonstrated that exposure to manganese, another divalent metal, can have significant effects on brain tissue levels of GABA and glutamate (Fitsanakis et al., 2006). Lead has also been demonstrated to alter glutamate release and uptake in various brain regions (Lasley and Gilbert, 2002; Struzynska et al., 2005). We did not measure transmitter release or transporter levels, so it is unclear if uranium is producing similar effects, but uranium clearly does not alter total tissue levels of GABA or glutamate.

Chronic exposure to implanted uranium did not alter the number of striatal D2 dopamine receptors, hippocampal NMDA receptors, or cortical nicotinic Ach receptors. Exposure to other metals has been shown to produce changes in receptors. These effects have typically been observed at higher tissue metal concentrations than were obtained in the present study. At relatively low tissue concentrations, uranium does not appear to affect neurotransmitter receptor numbers. We did not measure affinity or function in these assays, however, the lack of effect on behavioral endpoints suggests that there were not subtle functional effects on neurotransmission either.

Detailed neuropathologic study failed to detect lesions attributable to DU and/or stress exposure. There was renal accumulation of uranium at six months, with the high dose group reaching

concentrations similar to those seen in the low-dose soluble DU exposed rats in the acute study, but without the renal injury seen in the latter animals.

In summary, this study demonstrated that pelleted uranium can be mobilized from an intramuscular site, and enter and remain in brain tissue, where its major long-term effect was reduction in some neurotransmitters, most notably striatal dopamine. Stress did not alter this effect.

F. *Ancillary Studies Outside Statement of Work*

1. Co-Principal Investigator, Dr. David Barber, gave the presentation "Neurological and Behavioral Effects Following Co-Exposure to Uranium and Stress" to the Department of Veterans Affairs Research Advisory Committee on Gulf War Veterans' Illnesses, Washington D.C., in 2005. Dr. Barber also discussed the implications of this work with a view to its relationship with the spectrum of Gulf War illnesses being encountered in patients in VA medical facilities (from Year 4 Annual Report).
2. There was a potential collaboration with Dr. Michael Aschner, Vanderbilt University Medical Center. This involved assays of divalent metal transporter-1 (DMT-1) levels, a measure of potential uranium transport from blood to brain. We supplied Dr. Aschner with frozen (-70° C) samples from the cerebral cortex, striatum, hippocampus and cerebellum from rats sacrificed for neurochemistry in the Acute Study (Task 4, above). However, due to his recent move from Wake Forest University to Vanderbilt, short staffing in his laboratory, and an absence of funding for this work, Dr. Aschner's group has been unable to perform these assays. This may be possible in the future. We do maintain a parallel set of frozen brain samples from the Long-Term Study (Task 5, above), in the event it becomes possible to have DMT-1 assays done on this material (from Year 4 Annual Report).
3. During the project, a collaborative study was done with Dr. Ron Mervis, Neurostructural Research Laboratory, University of South Florida, Tampa, FL. Data from this work has been noted in the preceding sections for Task 3 - Pilot Stress Study and Task 5 - Long-Term (Chronic) Toxicity Study. The work consisted of Golgi staining and evaluation of basilar dendrites of hippocampal CA_1 pyramidal neurons.
4. Renal Toxicity - Although not part of the original study design, we assessed DU-related renal toxicity. This appeared to be an issue in the Acute Toxicity Study (Task 4). Thus we pursued renal DU toxicity in both the Acute Toxicity Study (see Task 4 above and Zimmerman et al. manuscript in the Appendix) and in the Long Term (Chronic) Toxicity Study (see Task 5, above). In addition, the

Tolson et al. paper (Toxicology 206: 59-73, 2005 included in Year 4 [10/2005] Annual Report) addresses mechanistic issues in DU-induced renal injury.

5. Uranium cytotoxicity in cultured dopaminergic neurons – Given the neurochemical findings of Tasks 4 and 5, we initiated a study of uranium toxicity in cultured neurons. This showed that uranium is cytotoxic to N27 rat mesencephalic dopaminergic neurons, however concentrations of greater than 250μM are required to produce significant toxicity under the conditions tested (Figure 11). To examine the mechanisms by which uranium speciation affects toxicity in N27 cells, cells were exposed to uranium in serum-free media with various concentrations of bicarbonate and phosphate. The uranyl ion readily forms complexes with bicarbonate and phosphate and these complexes are the dominant forms of low molecular weight uranium in most extracellular fluids (Sutton and Burastero, 2004). In media containing 20mM bicarbonate, uranium toxicity was greatly enhanced by the presence of phosphate. This suggests that the uranyl phosphate complex, which is of low solubility, may be the toxicologically important form for uranium neurotoxicity. This is consistent with work by Muller et al. (Muller et al., 2006) which demonstrated that uranium nephrotoxicity is primarily due to uptake of uranyl phosphate through the sodium-phosphate co-transporter. However, in media containing 50mM bicarbonate, the presence of phosphate was protective. Additionally, phosphonoformic acid, a sodium-phosphate cotransporter inhibitor, had no effect on uranium cytotoxicity in N27 cells (data not shown). Neuronal cells express a variety of phosphate cotransporters, some of which bear little resemblance to the renal form. It seems that uranium uptake and toxicity in dopaminergic cells is not solely dependent on uranyl phosphate.

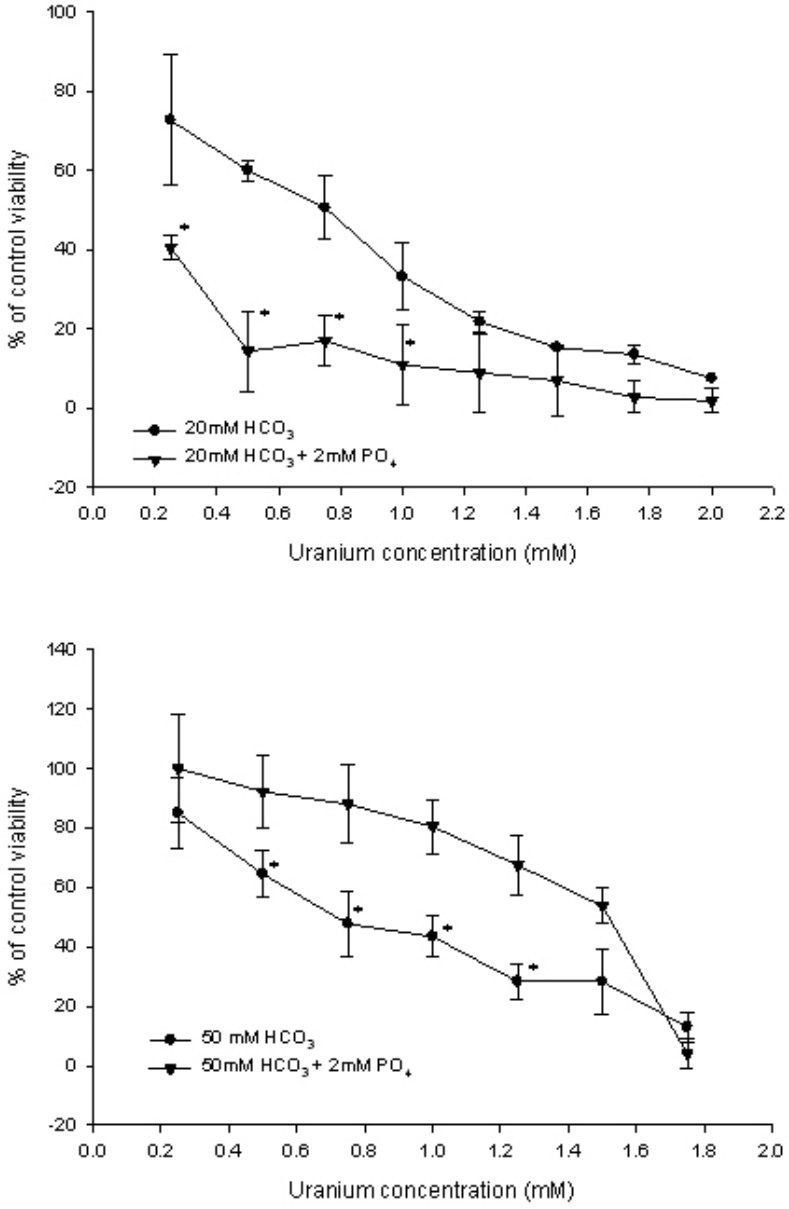

Figure 11. Cytotoxicity of uranium in N27 rat mesencephalic cells. Cells were exposed to uranium in serum-free media containing 20mM bicarbonate (A) or 50mM bicarbonate (B) with and without 2mM phosphate. Cell viability was determined by XTT reduction. Values are mean ± SD of three replicates. Each replicate was performed in 4 wells. * indicates values that are significantly different from the effect of uranium in the absence of phosphate ($p<0.05$).

II. Problems Encountered, Solutions and Recommended Approaches to Future Work.

A. Brain glutamate in Acute DU Toxicity Study (Task 4)- We were not able to obtain data on regional brain glutamate concentration in the acute study due to methodological problems with the HPLC. These issues were resolved prior to analysis of samples from the Long-Term DU Toxicity Study.

B. Assessment of dendritic change in the Long-Term DU Toxicity Study as seen using Golgi-stained preparations - As noted above under Task 5, we have utilized Golgi staining of hippocampi from tantalum control and high-dose DU exposed rats, with and without stress. By this procedure (performed by Dr. Ron Mervis, Neurostructural Laboratories, Tampa, FL) it was determined that exposure to DU altered the dendrites, most strikingly eliciting an increase in dendritic material in inner regions of the basilar dendrites of CA1 neurons. This has been presented at the 2006 meetings of the Society for Toxicology and Society for Neuroscience (see Mervis et al., Amato et al. Reportable Outcomes). This is provocative, since there is considerable public interest on possible neurotoxic effects related to DU exposure. To strengthen or disprove this finding, it would be helpful to be able to perform similar study on brains from the low and mid-dose DU-exposed rats, to determine if a dose-effect is present. Funds to do this were not available. Dr. Mervis estimates some $4,000 would be needed for this additional work to be done.

C. Publications - Publication of results from the project has lagged behind acquisition and interpretation of data. Thus, while papers for Tasks 1-3 are published or in press (see Reportable Outcomes- Manuscripts: Barber et al., 2005, Tolson et al. 2005, Hancock et al., in press), this has not as yet been done for Tasks 4 and 5. For Task 4 (Acute DU Toxicity Study), two manuscripts are in the late stages of preparation for submission to journals. These are included in the Appendix, and are:

1. Barber et al., Neurological effects of acute uranium exposure with and without stress. For submission to Toxicologic Sciences.
2. Zimmerman et al., Temporal clinical chemistry and microscopic renal effects following acute uranyl acetate exposure. For submission to Toxicologic Pathology.

The accepted papers will be submitted as a supplement to this report. Material from the Long-Term DU Toxicity Study will be assembled and published, with a focus on neurological and renal effects. Once accepted for publications, these manuscripts also will be submitted as a supplement to this report.

KEY RESEARCH ACCOMPLISHMENTS

- The Toxicokinetic Study of DU (Task 2) was reported in the Annual Report of Year 1 (10/2002). In summary this demonstrated that DU quickly entered the brain following intraperitoneal administration of 1 or 10 mg/kg. Brain concentrations ranged from 4-18 ng/g 8 hours after administration of 1 mg/kg. Stress at the time of exposure reduced concentrations of uranium in the cerebellum and hippocampus, and thus may have had an ameliorating effect.

- Pilot Stress Study (Task 3) results were presented in Year 3 Annual Report (10/2003). The data revealed there was elevation of plasma corticosterone and reduction in body weight across the study. These were noted in rats receiving daily swim stress and in those having restraint stress for four consecutive days followed by one day with swimming. The plasma corticosterone elevations were related to the swimming episodes. No significant elevations were noted in rats exposed to routine handling (control group) or daily restraint stress. This stress model was used in subsequent components (Tasks 4 and 5) of the project.

- Acute DU Toxicity Study (Task 4) results were presented in the Year 3 and 4 (10/2004, 2005) Annual Reports. In this study we used three dose levels of soluble DU (as uranyl acetate) given as a single intramuscular dose in the presence and absence of pre-dosing stress. There was DU dose-associated, transient reduction in dopamine content of the striatum. This was seen on post-dosing day 3, only at the highest (1.0 mg/kg DU) dose of this metal, with protection offered by the prior exposure to stress. Values returned to normal when studied on day 7. Exposure to UA did not alter DOPAC levels or numbers of D2 receptors in the striatum. No effect of DU or stress was observed on levels of GABA, serotonin, norepinephrine or GSH in the striatum, hippocampus, cerebellum, or cortex. Several clinical changes were noted in animals on the study, most prominently DU-associated reduced forelimb grip strength, ambulatory movement and body weight gain. These were most marked in the post-dosing period, with some recovery noted later in the study. These changes are paralleled by transient DU-dose-related acute tubular necrosis in the kidneys and uremia, features unaffected by stress. These results indicate that single intramuscular exposures to uranium produce sustained elevation of brain uranium levels and at doses above 0.3 mg/kg can have adverse neurological effects. However, uranium exposure also produced renal toxicity, so it is unclear if the neurological effects are a direct result of uranium or are secondary to renal injury.

- Long-Term DU Toxicity Study (Task 5) - This was a large, complex project, involving exposure to tantalum controls and three levels of implanted DU pellets, with and without stress. The latter was applied at intervals throughout the six-month duration of the study, and was effective, based upon serial transiently elevated plasma corticosterone levels, related to swim stress. Other stress-induced changes were body weight reduction and increases in ambulatory and

non-ambulatory motor activity. There were DU dose-related increased uranium concentrations in serum, kidney and brain regions in rats sacrificed six months post-exposure, unaffected by stress. Neurochemical alterations included decrease in dopamine in the striatum and epinephrine in the cerebellum, seen in the high DU dose group, and unmodified by stress. The dopamine effect parallels a transient finding in the Acute Study (see above). There was no change in striatal levels of DOPAC or homovanillic acid (HVA), major metabolites of dopamine. Exposure to implanted uranium pellets for 6 months with or without concurrent stress did not produce significant effects on reduced or total glutathione, GABA or glutamate concentrations in any brain region. Nor did it alter the number of striatal D2 dopamine receptors, hippocampal NMDA receptors, or cortical nicotinic ACh receptors. Detail neuropathologic study failed to detect lesions attributable to DU and/or stress exposure. There was renal accumulation of uranium at six months, with the high dose group reaching concentrations similar to those seen in the low-dose soluble DU exposed rats in the acute study, but without the renal injury seen in the latter animals. This study demonstrated that pelleted uranium can be mobilized from an intramuscular site, and enter brain tissue, where its major effect was reduction in striatal dopamine and cerebellar epinephrine, unmodified by ongoing stress exposures.

REPORTABLE OUTCOMES

Abstracts (also presented)

D.S. Barber and M.J. Kopplin. Regional distribution of uranium in rat brain. Toxicol. Sci. 72 (supplement): 19, 2003. (Society of Toxicology, 2003)

J.W. Munson, J.K. Tolson, B.S. Jortner, S.M. Roberts and D.S. Barber. Heat shock proteins and uranium nephrotoxicity. Toxicol. Sci. 72 (supplement): 347, 2003. (Society of Toxicology, 2003)

M. Pomeroy, B. Jortner, M. Ehrich, J. Robertson, and D. S. Barber. Uranium and cell death in the rat kidney. Toxicol. Sci. 72 (supplement): 16, 2003. (Society of Toxicology, 2003)

S.K. Hancock, M.F. Ehrich, J. Hinckley, T. Pung, K.L. Farris, B.S. Jortner. Stress and neurotoxicant exposure. Comparison of effects of several stress models on the acute neurotoxicity of the organophosphate chlorpyrifos. (Society for Neuroscience, 2003).

L. Tobias, D. Barber, K. Zimmerman, S. Hancock, J. Hinckley, M. Ehrich, B. S. Jortner. Nephrotoxic effects of depleted uranium (DU) in the rat. Interactions with stress. Toxicologist 84:34, 2005. (Society of Toxicology, 2005)

D.S. Barber, S.K. Hancock, A.M. McNally, J. Hinckley, E. Binder, M.F. Ehrich and B.S. Jortner. Neurological effects of acute uranium exposure. Toxicologist 84:123, 2005. (Society of Toxicology, 2005)

D.S. Barber, S.K. Hancock, J. Hinckley, E. Binder, K. Zimmerman, M.F. Ehrich and B.S. Jortner. Neurological effects of chronic uranium and stress exposure. Toxicologist 90:369, 2006. (Society of Toxicology, 2006)

R.E. Mervis, D.S. Barber, M. Ehrich, S. Hancock, J. Hinckley, J. Kotick, M. Shah, T. Amato, B.S. Jortner. Stress and depleted uranium exposure alter hippocampal dendritic morphology in the rat. Toxicologist 90:369, 2006. (Society of Toxicology, 2006)

T. Amato, J. Kotick, S. Gogak, B.S. Jortner, D.S. Barber, S. Hancock, M. Ehrich, J. Hinckley, M. Shah, R.F. Mervis. Exposure to depleted uranium results in CA1 dendritic neuroplasticity in the adult rat hippocampus. (Society for Neuroscience, 2006)

Presentations

D. Barber and B.S. Jortner. Presentation at Force Health Protection Review of Depleted Uranium Projects, Albuquerque, NM. Multifactorial assessment of depleted uranium neurotoxicity, December 2002.

B.S. Jortner. Presentation at the Bioscience Review 2004, Hunt Valley, MD. The effect of stress on the acute neurotoxicity of the organophosphate chlorpyrifos, May 2004.

D. Barber. Presentation to Department of Veterans Affairs Research Advisory Committee on Gulf War Veterans' Illnesses, Washington D.C. Neurological and behavioral effects following co-exposure to uranium and stress, 2005.

Manuscripts

D.S. Barber, M.F. Ehrich, B.S. Jortner. The effect of stress on the temporal and regional distribution of uranium in rat brain after acute uranyl acetate exposure. J. Toxicol. Environ. Health 68, 99-111, 2005.

J.K. Tolson, S.M. Roberts, B. Jortner, M. Pomeroy, D. Barber. Heat shock proteins and acquired resistance to uranium nephrotoxicity. Toxicology 206:59-73, 2005

S. Hancock, M. Ehrich, J. Hinckley, T. Pung, B.S. Jortner. The effect of stress on the acute neurotoxicity of the organophosphate insecticide chlorpyrifos. Toxicol. Appl. Pharmacol., in press.

D.S. Barber, S.K. Hancock, A.M. McNally, J. Hinckley, E. Binder, M.F. Ehrich, B.S. Jortner. Neurological effects of acute uranium exposure with and without stress (in preparation for Toxicological Sciences, included in Appendix)

K.I. Zimmerman, D.S. Barber, M.F. Ehrich, L. Tobias, S. Hancock, J. Hinckley, E.M. Binder, B.S. Jortner. Temporal clinical chemistry and microscopic renal effects following

acute uranyl acetate exposure. (in preparation for Toxicological Pathology, included in Appendix)

CONCLUSIONS

Major conclusions drawn from the project are as follows. The Toxicokinetic Study of DU (Task 2) was reported in the Annual Report of Year 1 (10/2002). In summary this demonstrated that DU quickly entered the brain following intraperitoneal administration of 1 or 10 mg/kg. Brain concentrations ranged from 4-18 ng/g 8 hours after administration of 1 mg/kg. Stress at the time of exposure reduced concentrations of uranium in the cerebellum and hippocampus, and thus may have had an ameliorating effect.

Conclusions for the six-week long Pilot Stress Study (Task 3) were presented in Year 3 Annual Report (10/2003). The data revealed there was elevation of plasma corticosterone and reduction in body weight across the study. These were noted in rats receiving daily swim stress and in those having restraint stress for four consecutive days followed by one day with swimming. The plasma corticosterone elevations were related to the swimming episodes. No significant elevations were noted in rats exposed to routine handling (control group) or daily restraint stress. This stress model was used in subsequent components (Tasks 4 and 5) of the project.

Conclusions for the Acute DU Toxicity Study (Task 4) were presented in the Year 3 and 4 (10/2004, 2005) Annual Reports, and are as follows. In this study we used three dose levels of soluble DU (as uranyl acetate) given as a single intramuscular dose in the presence and absence of pre-dosing stress. There was DU dose-associated, transient reduction in dopamine content of the striatum. This was seen on post-dosing day 3, only at the highest (1.0 mg/kg DU) dose of this metal, with protection offered by the prior exposure to stress. Values returned to normal when studied on day 7. Exposure to UA did not alter DOPAC levels or numbers of D2 receptors in the striatum. No effect of DU or stress was observed on levels of GABA, serotonin, norepinephrine or GSH in the striatum, hippocampus, cerebellum, or cortex. Several clinical changes were noted in animals on the study, most prominently DU-associated reduced forelimb grip strength, ambulatory movement and body weight gain. These were most marked in the post-dosing period, with some recovery noted later in the study. These changes are paralleled by transient DU-dose-related acute tubular necrosis in the kidneys and uremia, features unaffected by stress. These results indicate that single intramuscular exposures to uranium produce sustained elevation of brain uranium levels and at doses above 0.3 mg/kg can have adverse neurological effects. However, uranium exposure also produced renal toxicity, so it is unclear if the neurological effects are a direct result of uranium or are secondary to renal injury.

Long-Term DU Toxicity Study (Task 5) - This was a large, complex project, involving exposure to tantalum controls and three levels of implanted DU pellets, with and without stress. The latter was applied at intervals throughout the six-month duration of the study, and was effective, based upon serially elevated plasma corticosterone levels,

related to swim stress. Other stress-induced changes were body weight reduction and increases in ambulatory and non-ambulatory motor activity. There were DU dose-related increased uranium concentrations in serum, kidney and brain regions in rats sacrificed six months post-exposure, unaffected by stress. Neurochemical alterations included decrease in dopamine in the striatum and epinephrine in the cerebellum, seen in the high DU dose group, and unmodified by stress. The dopamine effect parallels a transient finding in the Acute Study (see above). There was no change in striatal levels of DOPAC or homovanillic acid (HVA), major metabolites of dopamine. Exposure to implanted uranium pellets for 6 months with or without concurrent stress did not produce significant effects on reduced or total glutathione, GABA or glutamate concentrations in any brain region. Nor did it alter the number of striatal D2 dopamine receptors, hippocampal NMDA receptors, or cortical nicotinic ACh receptors. Detailed neuropathologic study failed to detect lesions attributable to DU and/or stress exposure. There was renal accumulation of uranium at six months, with the high dose group reaching concentrations similar to those seen in the low-dose soluble DU exposed rats in the acute study, but without the renal injury seen in the latter animals. In summary, this study demonstrated that pelleted uranium can be mobilized from an intramuscular site, and enter brain tissue, where its major effect was reduction in striatal dopamine, unmodified by stress.

Recommendations for Future Work- These findings suggest that DU exposure impacts upon an important neurotransmitter system, both in the acute and long-term toxicity studies. This is of interest, since the exposure to DU was by two different methods and exposure rates. This needs to be confirmed. Material from the present project could be used to further evaluate this effect, employing immunohistochemistry for dopaminergic neurons and their processes. This would use primary antibodies to tyrosine hydroxylase in sections from the striatum and substantia nigra. This would not require additional animal dosing. In addition, more direct studies could be made using direct application of soluble DU to organ slices/cultures *in vitro*. Some of this has been initiated (see Ancillary Studies, above). Preliminary evaluation of Golgi impregnated hippocampal neurons from rats in the long-term DU toxicity study suggesting high dose of DU (the only one being studied) and stress related dendritic alteration is provocative. This part of the study needs to be extended to the mid- and low-doses of implanted DU, to provide definitive evidence of this effect. This is of importance given the role of that brain region in learning and memory. It must be admitted that there were no changes found in our studies of learning and memory in these rats using active and passive avoidance.

Overall Summary, Implications of the Research

The major findings of the project include the following. We developed and validated a militarily relevant stress model in rats. This consisted of episodic exposures to mild, routine (restraint) and severe, novel (forced swimming) stressors. The latter elicited marked but transient elevation in plasma corticosterone. This stress model was employed in subsequent acute and long-term DU studies in rats, to see if it modified the toxicity of the metal. Detailed clinical, neurochemical and neuropathological methods

were used to assess these effects. As regards these DU toxicity studies, we found that intramuscular exposure to soluble (one-time exposure to uranyl acetate) or pelleted DU (long-term exposure) resulted in absorption of uranium and its deposition in all brain regions examined (cerebral cortex, striatum, hippocampus and cerebellum). Multiple stress episodes during the exposure period did not affect this process. The major neurological effects of these DU exposures was on the dopaminergic system, in particular a decrease in dopamine content of the striatum, seen in both the acute and chronic studies. The mechanism of this effect on dopamine was not determined. No DU-associated lesions of the nervous system were detected, except for some alteration of the basilar dendrites of hippocampal CA1 neurons with long-term exposure to the high-dose of implanted DU. There were some DU-related effects in the acute study, such as decreased grip strength, ambulatory movement and body weight gain. Pre-dosing stress in part ameliorated the dopaminergic and grip strength effects of acute DU exposure. However, acute exposure to DU also produced renal toxicity, unaffected by stress, which may have been a factor in the toxicity.

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APPENDIX

STATEMENT OF WORK- Revised 1/15/05
Note- changes indicated in green type

The proposed work will use the male Sprague Dawley rat to characterize the kinetics and toxicity of depleted uranium (DU) in the brain. The ability of stress to affect disposition and toxicity of DU will also be examined, as stress can alter the permeability of the blood-brain barrier and enhance neurodegeneration. The studies would examine neurotoxicity of DU with and without stress in acute and long-term exposures. Assessment would include detailed morphological, neurobehavioral, neurochemical and toxicokinetic methods.

The experimental design will be a split plot design. The whole plot will be a factorial array in a randomized complete block design. The treatments are stress (2 levels- stressed and unstressed) and DU dose (2-4 levels including negative controls). The whole-plot is rat and the sub-plot is brain region (4 regions). For the **kinetic study**, a 2x2 design will be used (2 doses of DU and 2 levels of stress [stressed or unstressed]). The stressor will be applied once daily for at least 5 days prior to DU exposure. Uranium levels in cortex, hippocampus, caudate-putamen, and cerebellum, as well as in blood, will be determined at several times after DU exposure (e.g., 8 hours, 1 day, 7 days, and 30 days). For the **acute toxicity study**, a 2x4 design will be used, consisting of 2 levels of stress (stressed and unstressed) and 4 levels of DU (control, low, mid and high). For stressed animals there will be daily routine stress with superimposed novel stress just prior to dosing (Hancock *et al.*). At several times after DU exposure (e.g., 1, 3, 7 and 30 days), brain samples will be analyzed for neurotransmitter levels, receptor numbers, evidence of oxidative stress and regional uranium concentrations. At the later time periods (e.g. 3, 7 and 30 days), samples will also be taken for neuropathological study. Neurobehavioral testing will be performed before dosing and weekly thereafter. The acute toxicity study will be performed in a number of experimental blocks. For the **long-term toxicity study**, a 2x4 design will be used with 2 levels of stress (stressed and unstressed) and 4 levels of implanted DU (tantalum negative control, low, medium, and high concentrations of DU pellets). Stress will be induced by frequently (such as 5 days/week) applying a routine stressor with superimposed periodic novel stress throughout the study (using parameters confirmed by a prior **pilot stress study**). Animals will be exposed to DU for 6 months. Behavioral testing will be performed before dosing and every 3 weeks thereafter. After 6 months, samples will be taken for neurochemical, neuropathological and brain uranium concentration analyses, as noted above. All determinations will be made on 3-5 separate samples. The study will be performed in experimental blocks.

The entire project will be conducted at two institutions, Virginia Tech (B. Jortner, principal investigator and neuropathologist and M. Ehrich, neurotoxicologist) and the University of Florida (D. Barber, heavy metal toxicologist), using the following temporal plan.

Months 1-4: Preparations for study (both institutions) and preliminary studies to identify appropriate doses (University of Florida). Consultation on doses (Virginia Tech).

Months 5-12 (kinetic study): Kinetics of DU (as uranyl acetate) in the brain and interaction of stress and DU are studied using 4 treatments (low DU, high DU, low DU + stress, and high DU + stress) at 4 times (e.g. 8 hours, 1 day, 7 days, and 30 days) after a single injection of DU (University of Florida). Data analysis (both institutions).

Months 13-26 (pilot stress study and acute toxicity study): A pilot study of long-term routine stress with periodic superimposed novel stress will be done, using plasma corticosterone levels as a measure. An acute study will be undertaken, assessing toxicity and uranium kinetics of a single intramuscular injection of DU (as uranyl acetate). This will employ 8 treatments (vehicle control, low DU, mid DU, high DU, control + stress, low DU + stress, mid DU + stress and high DU + stress) at 4 times (e.g. 1, 3, 7, and 30 days after injection). Procedures include: in-life study, neurobehavioral assessment, neuropathology (Virginia Tech); tissue uranium assays (University of Florida); neurochemistry, data analysis (both institutions).

Months 27-35 (acute toxicity study): The in-life portions of the acute study are completed, and assessment and analysis of samples collected continues.

Months 36-48 (acute and log-term toxicity studies): In-life portion of long-term (6 month exposure) study is conducted, with assessment of toxicity and uranium kinetics following intramuscular injection of DU pellets. Eight treatments are used, tantalum control, low DU, medium DU, high DU, tantalum + stress, low DU + stress, medium DU + stress, and high DU + stress. Procedures include: in-life study (stress, neurobehavioral assessment), neuropathology (Virginia Tech), uranium assays (University of Florida), neurochemistry, data analysis (both institutions). Evaluation of samples from the acute study continues.

Months 49-60 (acute and log-term toxicity studies, sample and data analysis): Analysis of samples from the acute and long-term studies is completed, with associated statistical evaluation, and assembly of results and preparation of final report (both institutions).

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Bernard S. Jortner, VMD
Professor of Pathology, Principal Investigator

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Virginia Tech Office of Sponsored Programs

METHODS NOT PREVIOUSLY REPORTED

Amino acid neurotransmitter analysis

GABA and glutamate were quantified by reversed phase HPLC with electrochemical detection after derivatization with o-phthaldehyde as described by Peinado et al. (Peinado et al., 1986). Samples were diluted with 0.1M perchloric acid prior to derivatization to obtain concentrations that were in the linear range of the analytical method. Following analysis, data was adjusted for dilution and normalized to wet weight of brain tissue that was initially homogenized.

Total glutathione analysis

At sacrifice, samples were homogenized in xxx and frozen at -80°C until analysis. Total glutathione, which is the combination of reduced and oxidized glutathione, was determined by the enzyme recycling assay as described by Sian et al. (Sian et al., 1997). Data was normalized to wet weight of brain tissue used to prepare the initial homogenate.

NMDA receptor number

Hippocampi were dissected immediately after euthanasia and frozen on dry ice. Samples were stored at -80°C until analysis. Samples were thawed and homogenized in 0.32M sucrose, 10mM Tris, pH 7.6 followed by centrifugation at 1000xg for 10 minutes to remove large debris. The supernatant was centrifuged at 20,000 xg for 20 minutes to pellet membrane fragments. This pellet was resuspended in 5mM Tris-HCl, pH 7.6 and centrifuged at 40,000 x g to remove endogenous neurotransmitters. Pellet was washed again in 5mM Tris-HCl and then resuspended in 5mM Tris-HCl, pH 7.6. Membrane protein was determined by the BioRad protein assay using bovine albumin as a standard. NMDA receptor numbers were determined from the binding of 10nM ^{3}H MK-801 which was determined to be a saturating concentration. Non-specific binding was determined in the presence of 5µM unlabelled MK-801. Assays were conducted as described by Guilarte and Miceli (Guilarte and Miceli, 1992).

Nicotinic acetylcholine receptors

Cortical membrane preparations were used to determine the number of nicotinic acetylcholine receptors present based on ^{3}H methylcarbamylcholine binding. Membranes were prepared and binding of 5nM 3H methylcarbamylcholine was determined as described previously (Anderson and Arneric, 1994). Nonspecific binding was determined in the presence of uM unlabelled nicotine.

Cytotoxicity of uranium in cultured neuronal cells

N-27 rat dopaminergic mesencephalic neuronal cells were a generous gift of Dr. Bin Liu (University of Florida). N-27 cells were maintained in RPMI-1640 containing 10% heat inactivated fetal bovine serum and 100U/ml penicillin, 100µg/ml streptomycin and 2.5µg/ml amphotericin B. Cells were grown in humidified incubators at 37°C with 5% CO_2. For cytotoxicity assays, 4 x 10^3 cells were plated into each well of 96-well tissue culture plates in growth media. Cells were allowed to attach overnight and media was replaced with serum-free minimal essential media (MEM) that contained no phosphate or bicarbonate. Uranyl bicarbonate stocks were prepared by dissolving uranyl acetate in sodium bicarbonate solution and adjusting pH to 7.2. To determine the role of bicarbonate and phosphate complexes in uranium neurotoxicity, cells were exposed to uranium in serum-free MEM that contained either 20 or 50mM bicarbonate ion and 0 or 2mM phosphate ion. Cells were treated with concentrations of uranium from 0.1 to 2mM for 24 hours. Cell viability was determined by XTT reduction based on absorbance at 450nm as described previously (Tolson et al., 2005).

SUMMARY OF RAW MEANS AND P-VALUES FOR SELECTED RESPONSE VARIABLES NOT REPORTED IN PREVIOUS ANNUAL REPORTS

Acute Depleted Uranium Study

Passive Avoidance (*p=0.04)
Table 1A. P-values from passive avoidance latency.

Effect	Latency
Stress	0.6284
DU	0.3840
Stress*DU	0.6589
Study Day	**<.0001**
Stress*Study Day	0.7322
DU*Study Day	0.6866
Stress*DU*Study Day	0.0765

Table 2A. P-values from passive avoidance latency (test effect of slices). Day 0 is the first day of exposure to stress. Animals were dosed with DU on Day 4.

Effect	Stress	Study Day	Latency
DU*Stress*Study Day	non-stressed	12	0.6867
DU*Stress*Study Day	non-stressed	19	**0.0401**
DU*Stress*Study Day	non-stressed	26	0.2451
DU*Stress*Study Day	non-stressed	31	0.6385
DU*Stress*Study Day	stressed	12	0.2067
DU*Stress*Study Day	stressed	19	0.9625
DU*Stress*Study Day	stressed	26	0.9131
DU*Stress*Study Day	stressed	31	0.2408

Table 3A. Passive Avoidance Latency. Each value is the mean ± SEM (based on the pooled MSE), n=10-15 observations

DAY 12

Day	Stress	DU Dose	Latency (sec)
12	Non-stressed	0	165.16 ± 13.27
		low	173.03 ± 14.06
		mid	154.29 ± 10.61
		high	169.45 ± 11.46
	Stressed	0	179.44 ± 10.25
		low	152.55 ± 12.58
		mid	155.79 ± 10.61
		high	152.04 ± 11.02
19	Non-stressed	0	140.45 ± 21.56
		low	143.56 ± 22.86
		mid	112.97 ± 17.27
		high	71.27 ± 18.65*
	Stressed	0	125.33 ± 16.68
		low	130.05 ± 20.45
		mid	120.33 ± 17.27
		high	116.65 ± 17.93
26	Non-stressed	0	113.98 ± 21.53
		low	151.46 ± 22.83
		mid	126.55 ± 17.25
		high	93.18 ± 18.63
	Stressed	0	120.91 ± 16.66
		low	107.67 ± 20.43
		mid	120.26 ± 17.25
		high	127.15 ± 17.91
31	Non-stressed	0	138.88 ± 18.98
		low	136.50 ± 20.13
		mid	129.46 ± 15.20
		high	157.89 ± 16.42
	Stressed	0	129.55 ± 14.69
		low	130.26 ± 18.01
		mid	148.29 ± 15.20
		high	103.48 ± 15.78

CORRECTIONS TO PREVIOUS REPORTS

Annual Report 3 (pp. 36-41) and Annual Report 4 (pp.45-47):
Error: Catecholamine data (serotonin, dopamine, norepinephrine) was reported in ng/mg tissue
Correction: Catecholamine data is reported in nmol/mg tissue

Annual Report 4:
Error: Figure 9 legend (p.21) indicates n=9-10 observations
Correction: Figure 9 graphs presenting no stress/stress results, n=36-40 observations.

Error: Figure 10 legend (p.22) indicates n=9-10 observations
Correction: Figure 10 graphs presenting no stress/stress results, n=36-40 observations; graphs presenting DU treatment results, n=18-20 observations.

Error: Figure 11 legend (p.23) indicates n=13-15 observations
Correction: Figure 11 graphs presenting no stress/stress results, n=54-60 observations; graphs presenting DU treatment results, n=27-30 observations. Body weights were determined for all animals.

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B. Jortner. Presentation at the Bioscience Review 2004, Hunt Valley, MD. The effect of stress on the acute neurotoxicity of the organophosphate chlorpyrifos, May 2004.

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K.I. Zimmerman, D.S. Barber, M.F. Ehrich, L. Tobias, S. Hancock, J. Hinckley, E.M. Binder, B.S. Jortner. Temporal clinical chemistry and microscopic renal effects following Acute uranyl acetate exposure. Toxicol Pathol (in preparation)

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ABBREVIATIONS USED IN THE REPORT

5-HIAA – 5-hydroxyindolacetic acid
ACh - acetylcholine
CORT – corticosterone
DOPAC – dihydroxyphenylacetic acid
DU - depleted uranium
F-Jade - Fluoro-Jade
FOB - Functional Observational Battery
GABA - γ aminobutyric acid
GFAP - glial fibrillary acidic protein
H&E - hematoxylin and eosin
HVA – homovanillic acid
n – number
nACh – nicotinic acetylcholine
NMDA – N-methyl-D-aspartate
t. blue - toluidine blue and safranin stain

Abstract of paper presented at the 2006 Society for Neuroscience Annual Meeting

EXPOSURE TO DEPLETED URANIUM RESULTS IN CA1 DENDRITIC NEUROPLASTICITY IN THE ADULT RAT HIPPOCAMPUS.

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Gulf War I veterans with long-term residual depleted uranium (DU) shrapnel were found to have lowered performance efficiency in selected neurocognitive tests. Studies of rats with intramuscular implantation of depleted uranium (DU) reveal that in addition to accumulation of this metal in kidney and bone, it is also increased in brain. This study was undertaken to assess the neurotoxicologic effects of implanted DU in adult rats, focusing on dendritic changes to the hippocampal pyramidal cells that could affect memory. Adult male Sprague-Dawley rats each had 20 DU or tantalum (control) pellets (1mm x 2mm) implanted in the gastrocnemius muscles, for a six month period. Upon sacrifice, coronal hemispheric blocks were removed and stained by the Rapid Golgi impregnation procedure. From coded slides, the basilar dendritic arbor of randomly selected hippocampal CA1 pyramidal cells was quantitatively assessed using Sholl analysis. Results show that -- relative to controls -- there was a significant **increase** (+23%, $p < .0005$, Wilcoxon test) in dendritic material in the inner 1/2 of the arbor in rats administered DU. There was no difference between groups for the outer half of the CA1 dendritic arbor. Thus, in adult rats, 24 weeks exposure to a high dose of implanted DU pellets caused an abnormal dendritic neuroplastic effect on CA1 branching and altered hippocampal circuitry. This may be the neuroanatomical basis for the deleterious cognitive sequelae described for the Gulf War soldiers.

NEUROLOGICAL EFFECTS OF ACUTE URANIUM EXPOSURE WITH AND WITHOUT STRESS

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Abstract

Uranium in the peripheral circulation has been shown to enter the brain and may cause adverse effects on the nervous system. In the current study, we examined the neurological effects of a single intramuscular injection of 0, 0.1, 0.3, or 1 mg uranium/kg (as uranyl acetate, UA) in the presence and absence of stress. Stress was applied for five days prior to uranium injection. Animals that were stressed had four-fold higher plasma corticosterone levels at the time of uranium exposure (763 ± 131 vs. 189 ± 91 ng/ml). Treatment with UA produced time and dose-dependent increases in serum and brain uranium levels. While serum levels returned to control levels by day 30, brain levels remained elevated. Prior stress did not affect the distribution or retention of uranium in the brain or serum. Exposure to uranium decreased ambulatory activity and weight gain, regardless of stress treatment. Exposure to 1 mg U/kg UA also decreased forelimb grip strength and transiently impaired working memory, but these effects were prevented by application of stress prior to uranium exposure. Rats treated with 1 mg U/kg exhibited a 30% decrease in striatal dopamine content 3 days after dosing (59 ± 6 vs. 41 ± 5 nmol/mg tissue), but levels returned to control 7 days after uranium exposure. The effect on dopamine was ameliorated by prior application of stress. Exposure to UA did not alter DOPAC levels or numbers of D2 receptors in the striatum. No effect of DU or stress was observed on levels of GABA, serotonin, norepinephrine, or GSH in the striatum, hippocampus, cerebellum, or cortex. These results indicate that single intramuscular exposures to uranium produce sustained elevation of brain uranium levels and at doses above 0.3 mg/kg can have adverse neurological effects. However, uranium exposure also produced renal toxicity, so it is unclear if the neurological effects are a direct result of uranium or are secondary to renal injury.

Introduction

Enrichment of uranium removes most ^{234}U and ^{235}U, producing depleted uranium (DU) which is approximately 99.7% ^{238} U. Depleted uranium has been used for counterweights in airplanes and missiles, as radiation shielding, and in inertial guidance devices. Due to its density and ability to undergo phase transition, depleted uranium is also used for anti-tank armor penetrators and as tank armor. The use of depleted uranium (DU) munitions in military operations has increased the potential exposure of military and civilian personnel to uranium (May et al., 2004). Exposure can occur from handling materials made of DU, inhaling or ingesting dust produced by the firing and impact of DU rounds, or having DU shrapnel fragments embedded in tissue. Soldiers deployed in Bosnia had elevated levels of urinary uranium compared to non-deployed personnel, but the observed levels were not outside the normal range (May et al., 2004). There are also a cohort of soldiers who were wounded with DU shrapnel that were not removable. These soldiers continue to exhibit elevated urinary uranium levels demonstrating ongoing uranium exposure (McDiarmid et al., 2001; McDiarmid et al., 2006). The consequences of depleted uranium exposure are unclear. Studies indicate that most toxicity associated with depleted uranium is due to chemical toxicity and the radioactive contribution is very small (Hartmann et al., 2002). Uranium-induced renal toxicity has been well documented (Diamond et al., 1989; Leggett, 1989), but the neurological effects of uranium exposure are poorly understood.

Access of polar molecules, such as uranium, to the central nervous system is primarily regulated by transport across the blood-brain barrier (BBB) and the blood-cerebrospinal fluid

(CSF) barrier (Zheng et al., 2003). Previous studies have demonstrated that uranium in blood rapidly enters the brain (Lemercier et al., 2003; Barber et al., 2005) and accumulates preferentially in the caudate putamen and hippocampus of exposed rats (Barber et al., 2005). This is consistent with data observed following long term exposure to uranium pellets, which demonstrated accumulation of uranium in the brain (Pellmar et al., 1999). Therefore, uranium clearly has access to the central nervous system, but does it cause adverse effects?

Previous work suggests that long-term exposure to DU can cause functional changes in hippocampal slices (Pellmar et al., 1999). Examination of soldiers wounded with DU shrapnel found a positive correlation between elevated uranium excretion and reduced performance on neurobehavioral testing in the absence of renal injury seven years after shrapnel exposure (McDiarmid et al., 2000). While these effects were not evident at later times, they raised questions about the potential for DU-induced neurotoxicity (McDiarmid et al., 2001; McDiarmid et al., 2002). Several recent studies have examined the effects of uranium on memory, dopamine metabolism, activity and sleep (Houpert et al., 2004; Belles et al., 2005; Lestaevel et al., 2005). However, there have been no comprehensive studies on the neurological effects of uranium exposure.

Stress produces many physiological changes in organisms and prolonged stress can itself cause neurological problems (Luine et al., 1994). While controversial, studies have also linked acute stress to increased permeability of the BBB (Friedman et al., 1996). Because combat situations are undoubtedly stressful, stress should be considered to accurately depict transport of uranium in the CNS during wartime military operations. In this study, we examined functional,

biochemical and pathological endpoints associated with the nervous system of normal and stressed animals to determine if there were adverse effects from a single dose of soluble uranium. Our results indicate that a single intramuscular exposure to soluble uranium increased brain uranium concentrations for up to 30 days. This exposure is sufficient to produce functional and biochemical changes that also persist for up to 30 days after exposure. However, all tested doses of DU tested produced some degree of renal injury which must be considered when evaluating the results.

Materials and Methods

Materials- Uranyl acetate dihydrate was purchased from Electron Microscopy Sciences (Ft. Washington, PA). This material contained 0.4% ^{235}U based on manufacturer data. Ultra-trace metal grade nitric acid (Optima, Fisher) and hydrogen peroxide (Ultrex II, JT Baker) were purchased from Fisher Chemical Company (Atlanta, GA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and were of the highest purity available.

Uranium and stress exposures

The study is a randomized complete block designed with a 2X4 factorial array treatment structure with 2 levels of stress (present or absent) and 4 levels of depleted uranium (0, 0.1, 0.3, or 1.0 mg U/kg). Five replicate experimental blocks were conducted. In each experimental block, each of 8 treatment groups were included with 3 animals/treatment group to give n=1 animal/treatment group/endpoint and an n=5 animals/treatment group/endpoint for tissue

uranium concentration, neurochemistry and neuropathology. For behavioral endpoints, there were 10-15 animals per treatment group.

Male Sprague-Dawley rats (250-320 g) were randomly assigned to 8 treatment groups that received various combinations of stress and uranium. Rats were housed in temperature and humidity controlled environment with 12 hour light:dark cycle. Animals were allowed ad libitum access to rodent chow (Harlan Tek-Lad 2018) and tap water.

Stress was induced by a combination of four consecutive days of restraint stress followed by swim stress on the fifth day. For restraint stress, each animal was placed in an individual Plexi-glas® tube (6 cm diameter X 22 cm long) with adequate breathing holes for a 30-minute period and then returned to its home cage (Konarska et al., 1989). For swim stress, each animal was placed in a 4-chambered tank of water (15" X 6"/ chamber, depth 32 cm) at 23°C - 25°C and allowed to swim for a 30-minute period. When swimming was completed, the animal was towel-dried for 1-2 minutes, returned to home cage and placed under a warming light. Animals that were not stressed were handled daily by removing the animal from its home cage, placing it in a box and immediately returning it to its home cage. Immediately after cessation of swim stress, animals were anesthetized with isofluorane and blood was collected from the orbital sinus for evaluation of corticosterone. While still under anesthesia, rats were treated with uranyl acetate in saline (0.33 ml/kg) by intramuscular injection into the hamstring muscle at doses of 0.1, 0.3, and 1.0 mg uranium/kg. Control rats were injected with 0.33 ml/kg of sterile saline. After dosing, rats were returned to their home cages and allowed *ad libitum* access to food and water. Animals were killed by carbon dioxide inhalation 1 day, 3 days, 7 days, or 30 days after

treatment. After euthanasia, animals were perfused with cold heparinized saline to eliminate blood contamination of tissue. Brains from animals used for biochemical or metal analysis were rapidly removed and dissected while on chilled platform. Cerebellum, cerebral cortex, striata and hippocampi were removed, placed into preweighed tubes and snap frozen. All samples were stored at -80°C until analysis. All procedures involving live animals were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (IACUC).

Corticosterone analysis

Blood was collected from the orbital sinus immediately following the stress session while rats were under isofluorane anesthesia. Whole blood was collected in heparinized microcentrifuge tubes, centrifuged @12000 rpm at 4° C for 4 minutes. The plasma was removed and frozen at -70° C until analysis by a corticosterone ^{125}I-radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA). Each sample was analyzed in duplicate and the mean value was used for further analysis.

Functional Observation Battery (FOB), Motor Activity, and Passive Avoidance

The clinical evaluations performed were as described by Ehrich et al. (2004) on all rats scheduled for sacrifice at 30 days post-dosing (n=10-15/treatment group). All observations and tests were conducted during the rat's active (dark) cycle. FOB and motor activity were assessed prior to dosing and at seven day intervals throughout the study.

The functional observational battery (FOB) used was that suggested by the U.S. Environmental Protection Agency (EPA) guidelines (US EPA, 1998). The FOB was conducted in the afternoon and the sequence of the examination was arranged to progress from the least to the most interactive with the animal. Following home cage and handling observations, the rat was placed in an open field for 3 minutes of undisturbed observations. The rat was then evaluated for reflexive responses (approach, touch, click, tail pinch, tail-limb and righting), followed by a number of measurements to determine the physiological state of the animal (body weight and temperature, accelerating rotarod [Columbus Instruments], grip strength and landing foot splay).

The horizontal motor activity level of animals was measured using an automated cage rack photobeam activity system (San Diego Instruments), which provides measurements of non-ambulatory or fine movements by counting repeated breaks of the same photobeam, ambulatory or large locomotor movements by counting breaks of adjacent photobeams and total activity by counting all photobeam breaks. Rats were placed in novel cages and activity measurements were collected for 30 minutes at two-minute intervals.

Memory was assessed using an automated step-through light-dark passive avoidance response system (Gemini Avoidance System, San Diego Instruments). A single acquisition (training) trial was performed prior to dosing. The trial consisted of a 30-second adaptation in a darkened chamber followed by the onset of a bright light. To avoid the light, the rat crossed over into a darkened adjacent chamber and received a mild 0.8 mA footshock for 1 second. The time to cross over from the light chamber to the darkened chamber (latency) was automatically

recorded with a maximum trial length of 180 seconds. The rats were tested for recall 24 hours after the acquisition period to determine whether they had been trained to criterion by remaining in the light chamber for 180 seconds to avoid the footshock. Any rat that did not meet the criterion was retrained and treated as a subgroup for data analysis. Retention trials, which mimic the training trials but without the footshock, were conducted at 7-8 day intervals throughout the study and the latency recorded was used to measure the ability of the rats to remember adverse stimuli.

Glutathione analysis

Tissue samples for glutathione analysis were initially homogenized in ice-cold 0.1M perchloric acid. Homogenates were centrifuged for 10 minutes at 14,000 xg and 4°C. 150μl of the supernatant was placed in a fresh tube containing 15μl of 1mM □-glutamylglutamate (as internal standard), 15μl of 10mM bathophenanthrolinedisulfonic acid (BPDS), and 30μl of 70% perchloric acid. Samples were stored at -80°C until analysis. Reduced and oxidized glutathione levels were determined in individual brain regions by HPLC analysis ((Fariss and Reed, 1987).

Neurotransmitter analysis

Samples for neurotransmitter analysis were homogenized in 0.1M perchloric acid containing 1mM isoproterenol as internal standard, centrifuged at 14,000 xg for 10 minutes at 4°C. For catecholamine analysis, 150μl of the supernatant was placed into a tube containing 2.5μl of 15mM EDTA and 2μl of 150mM ascorbic acid. Samples were stored at -80°C until analysis. Monoamine neurotransmitters were determined from perchloric acid extracts by reversed phase

HPLC using electrochemical detection (Zaczek and Coyle, 1982). Amino acid neurotransmitters were determined by reversed phase HPLC of o-phthalaldehyde derivatives using electrochemical detection (Peinado et al., 1986).

Dopamine D2 receptor analysis

Immediately after dissection, intact pieces of tissue were snap frozen with dry ice/ethanol bath and stored at -80°C. For analysis, samples were thawed and crude membrane fractions were prepared as previously described by Lepiku et al. (1997). Briefly, samples were thawed on ice and homogenized in 10 volumes of buffer (0.32M sucrose, 10mM HEPES, pH 7.4, and 2mM EDTA). Homogenate was centrifuged at 1000xg for 15 minutes to remove nuclei and cell debris and the supernatant S1 was centrifuged at 200,000 xg for 15 minutes. The resulting pellet was resuspended in homogenization buffer and centrifuged at 200,000 xg for 15 minutes. Pellet contained crude membrane fraction and was suspended in lysis buffer (50mM HEPES pH 7.4, 2mM EDTA). D2 receptor numbers were determined by saturation binding of 3H-raclopride. 5nM 3H-raclopride was added to a tube containing 25 ug of membrane protein and the mixture was incubated at 25°C for 3 hr. Incubations were terminated by filtration through glass fiber filters (Whatman GF/B) treated with 0.3% polyethyleneimine followed by 3 washes with ice-cold incubation buffer. Radioactivity retained on the filters was determined by scintillation counting. Non-specific binding was determined from samples containing 1μM (+)-butaclamol. Specific binding was determined as the difference between total binding and non-specific binding. Receptor number was determined from B_{max} values.

Uranium analysis

Blood samples were collected from the inferior vena cava into serum separator tubes (Becton-Dickinson, Franklin Lakes, NJ). Serum was obtained by centrifugation according to manufacturer's instructions. Subsamples of these serum collections were also used for BUN analysis (see below). Brains were removed immediately and frontal cortex, cerebellum, striatum, and hippocampus were isolated by blunt dissection. Samples from both hemispheres were pooled to provide adequate amounts of tissue for analysis. Samples were frozen at -80°C until analysis.

Samples were digested 0.5 ml of concentrated ultra pure nitric acid (Fisher, Optima) at 140ºC for 2 hours, followed by addition of 0.5 ml of 30% Ultrex II hydrogen peroxide (J.T. Baker) and further digestion at 110°C for 60 minutes. Samples were then quantitatively diluted to 5 ml with plasma grade water (Fisher) and filtered (0.45□m) prior to analysis.

Uranium analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS) by the Analytical Section of the Hazard Identification Core in the Southwest Hazardous Waste Program at the University of Arizona (Tucson, AZ). Analysis was conducted on an HP 7500a ICP-MS using iridium as an internal standard. Uranium concentration was determined from a standard curve of uranium based on the m/z 238 signal. Five repetitions were performed per sample and the average used to calculate uranium concentration. The limit of quantitation of this method was 0.002 ppb. Recovery was determined from samples spiked with 0.1-10 ppb uranium and determined to be 94-111%.

Blood Urea Nitrogen (BUN) Analysis

BUN was determined in serum samples obtained from the vena cava at euthanasia (see above). BUN was measured within one hour of sample collection Olympus AU400 clinical analyzer (Zimmerman et al, submitted).

Histopathology

On days 3, 7 and 30 after uranium dosing, rats from each treatment group were anesthetized with intraperitoneal pentobarbital sodium. They were then systemically perfusion-fixed with a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer. Following perfusion-fixation, cross-sections of medulla, and spinal cord (cervical, thoracic and lumbar), peripheral nerve (sciatic, tibial, sural, vagus), optic nerve, and longitudinal sections of dorsal root ganglion and associated spinal nerve roots were embedded in Polybed epoxy resin, sectioned at 1 μm thickness and stained with toluidine blue and safranin for light microscopic study. Cross-sections of the frontal, parietal, and occipital levels of the cerebral hemispheres, midbrain, cerebellum and pons, and kidney and longitudinal section of the ocular globe (rostro-caudal plane) were embedded in paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin (H&E). Brain sections as noted below were also stained with Fluoro-Jade B, a fluorescent stain to detect degenerating neurons (Schmeud and Hopkins, 2000), or immunostained for glial fibrillary acidic acid (GFAP). GFAP immunostaining employed the peroxidase-antiperoxidase method (Sternberger Monoclonals. Lutherville, MD). GFAP immunostaining was used for rats from Groups 1 (negative control), 4 (high dose DU), 5 (stress control), 8 (stress plus high dose DU) sacrificed on days three and seven. The Fluoro-Jade stain for neuronal degeneration (Schmeud and Hopkins, 2000) was employed on brains from selected

rats from these groups sacrificed on day three. The H&E and GFAP stained sections were examined by light microscopy. The Fluoro-Jade stained sections were studied using a Nikon epifluorescent microscope with an excitation filter at 450-490 nm and a barrier filter at 520 nm. In the absence of lesions in the high dose depleted uranium-exposed animals, the mid and low dose groups were not examined.

Statistical Analysis

Repeated measures ANOVA (RMANOVA) was used to analyze data from the functional observational battery and passive avoidance testing. Serum corticosterone values were analyzed by paired t-test. Neurotransmitter, glutathione, dopamine receptor number, uranium content and serum chemistry values were analyzed with PROC MIXED in SAS System (v. 8.02, SAS Institute, Cary, NC). Post-hoc analysis was performed by Tukey's test for all pairwise comparisons. P values <0.05 were considered to be statistically significant.

Results

Effect of stress on corticosterone levels

The application of episodic stress produced a 4-fold increase in circulating corticosterone levels at the time of uranium administration. Corticosterone levels increased from 189 ± 91 ng/ml in controls to 763 ± 131 ng/ml in stressed animals.

Brain uranium content

Intramuscular exposure to uranyl acetate resulted in increased levels of uranium in all tissues measured. Tissue uranium concentrations varied significantly by treatment group, day after treatment and tissue. Serum from unstressed animals treated with saline contained 0.06 ± 0.008 ng U/ml. Cerebral cortex, cerebellum, striatum, and hippocampus from these animals contained 0.08 ± 0.06, 0.08 ± 0.05, 0.11 ± 0.08, and 0.32 ± 0.24 ng U/g tissue, respectively. In all tissues sampled, uranium content increased with increasing dose (Figure 1). Plasma concentrations varied significantly across time, but in all treatment groups serum uranium returned to control levels by day 30. Rats treated with 0.1 mg U/kg were only analyzed twenty-four hours after uranium administration. Treatment with 1 mg U/kg resulted in delayed peaks in plasma levels, with the highest levels observed 7 days after administration. Treatment with 0.3 mg U/kg produced peak plasma levels 3 days after uranium exposure. Application of stress prior to uranium exposure did not significantly affect serum uranium levels (Figure 1A). In unstressed animals, cerebral cortical and cerebellar uranium levels peaked on day 3 after uranium exposure

and declined slightly thereafter in animals treated with 0.3 or 1.0 mg U/kg. The application of stress prior to uranium exposure prevented the rise in tissue uranium seen at three days post-dosing, though differences were only significant in cerebellum of rats treated with 0.3mg U/kg. However, stress increased the levels of uranium present in cerebral cortex 30 days after treatment with 1 mg U/kg (Figure 1). In hippocampus and striatum, uranium levels in unstressed animals tended to increase with time, but only reached significance in hippocampus of animals treated with 0.3 mg U/kg. Stress did not produce a significant effect on striatal or hippocampal uranium levels at any of the times measured. In all brain regions, tissue uranium levels remained elevated at 30 days after intramuscular exposure to either 0.3 or 1.0 mg U/kg (Figure 1).

Effect of Uranium on Behavioral Endpoints.

Intramuscular injection with uranyl acetate produced dose-dependent decreases in weight gain during the first 6 days after exposure (Table 2). Past day 6, rats in all treatment groups gained weight at similar rates. Application of stress prior to uranium exposure did not alter the effect of uranium exposure on weight gain. Body weights 30 days after uranium exposure were not significantly different among all treatment groups.

Administration of a single intramuscular injection of 0.3 or 1 mg uranium/kg decreased ambulatory activity in both stressed and unstressed rats at all times examined (Table 1). The magnitude of the effect was greatest at day 6 and decreased with time but remained statistically significant even at day 27. Stress alone did not have a significant effect on ambulatory motor

activity. No significant effect of uranium treatment or stress was observed on non-ambulatory or total motor activity (Table 1).

Uranium exposure also decreased forelimb grip performance. Increasing doses of uranium caused small, but significant, decreases in forelimb grip strength (Table 3). Administration of 1 mg/kg of uranyl acetate decreased forelimb grip strength in unstressed rats by 5-7% at all time points measured. The application of stress prior to uranium administration reduced the effect on forelimb grip strength to the point of non-significance. No effect of stress or uranium exposure was observed on hindlimb grip strength.

Treatment with uranium induced slight, but statistically significant, changes in arousal, response to click or pinch, and temperature when analyzed across all DU doses. Because the changes were very minor and showed no consistency with respect to dose or time, it is unlikely that they are of biological significance (See supplementary data, Table A1).

Effect of treatment on memory

There was a significant decrease in performance on the passive avoidance test for all animals over time which is expected following a single training period. Unstressed animals treated with 1.0 mg uranium/kg performed similarly to controls on day 12 but exhibited significantly poorer performance (decreased latency) on day 19 after dosing (Figure 2). These animals also tended to have poorer performance on day 26. Latency time returned to control levels by day 31 (Figure 2). A similar trend was observed in animals treated with 0.3 mg

uranium/kg. Animals that were stressed prior to uranium exposure exhibited similar performance as controls at all timepoints.

Effect of Uranium on Neurochemistry

Exposure to stress and/or uranium had no significant effect on GABA, serotonin, or norepinephrine levels at any time in any of the brain regions examined. Control levels of GABA were 1212 ± 148, 1089 ± 379, 2128 ± 414, and 1594 ± 616 nmol/g tissue in cerebral cortex, cerebellum, hippocampus, and striatum, respectively. Control levels of serotonin were 1.03 ± 0.10, 0.59 ± 0.17, 0.42 ± 0.16, and 1.36 ± 0.27 ng/mg tissue in cerebral cortex, cerebellum, hippocampus, and striatum, respectively. Control levels of norepinephrine were 1.99 ± 0.19, 1.44 ± 0.21, 2.62 ± 0.38, and 1.39 ± 0.46 ng/mg tissue in cerebral cortex, cerebellum, hippocampus, and striatum, respectively (see supplemental data for complete data).

Exposure to 1mg/kg uranium caused a transient depletion of dopamine in the striatum, where levels were reduced by 30% in unstressed animals 3 days after administration (Figure 3). In these animals, dopamine levels returned to normal by 7 days after dosing. This effect was not observed at lower doses of uranium in unstressed animals or in any of the stressed animals (Figure 3). The dopamine metabolite, DOPAC, was quantified in the striatum of unstressed rats receiving 1 mg U/kg to determine if increased dopamine metabolism is responsible for the decreases observed in dopamine levels in these animals. No significant change in DOPAC levels were observed in these rats at any time. DOPAC levels in control striatum were 8.1 ± 2. 7, 8.9 ± 2.6, 9.0 ± 4.9, and 9.7 ± 2.6 nmoles/ mg tissue at 1, 3, 7, and 30 days after treatment,

respectively. DOPAC levels in striatum of unstressed rats treated with 1 mg U/kg were 11.1 ± 4.7, 7.6 ± 1.3, 10.1 ± 2.9, and 9.7 ± 2.3 nmoles/mg tissues on days 1, 3, 7, and 30 after uranium exposure, respectively. To further explore the effect of uranium exposure on dopamine neurotransmission, the number of striatal dopamine D2 receptors was determined. No significant changes in the number of D2 receptors were observed in any treatment group at any time (Figure 4).

Brain glutathione

Glutathione levels were examined in striatum, hippocampus, cortex, and cerebellum as a marker of ongoing oxidative stress. No significant change was observed in response to uranium exposure, stress, or the combination at any timepoint or brain region examined.

Neuropathological effects of uranium exposure

No lesions of the nervous system were found in the brain using H&E, GFAP and Fluoro-Jade staining. In addition, 1 micrometer thick sections stained with toluidine blue and safranin did not demonstrate lesions in the medulla, spinal cord, dorsal root ganglion and several peripheral nerves. Given the neurochemical findings, dopaminergic regions such as the substantia nigra and striatum were given special attention, without detection of histologic alterations.

Renal Toxicity following uranium exposure

Exposure to all doses of uranium resulted in renal damage indicated by increases in serum BUN concentrations. BUN levels peaked on day 7 and had returned to control levels 30 days after uranium exposure (Table 4).

Discussion

This study demonstrated that there is efficient uptake and distribution of uranium to the brain following intramuscular exposure. Exposure to a single intramuscular dose of uranyl acetate increased serum and brain uranium levels for up to 30 days. Compared to previous work utilizing intraperitoneal injection, peak plasma levels were lower and achieved later following intramuscular injection, probably reflecting slower absorption from the muscle (Barber et al., 2005). Lower plasma levels of uranium also resulted in lower initial levels of uranium in all brain regions examined. This suggests that uranium transport into the brain was not saturated at higher plasma concentration and that there is a high capacity for uranium transport into the brain. In the current study, the highest levels of uranium in the brain were observed in the hippocampus of rats exposed to either 0.3 or 1.0 mg U/kg. This is consistent with previous studies of rats exposed by either intraperitoneal injection or implanted pellets and suggests that the pathways of uptake and distribution in the brain are similar for all exposure paradigms (Pellmar et al., 1999; Barber et al., 2005). Uranium elimination was very slow in all brain regions examined. Previous work demonstrated rapid initial elimination of uranium from the brain followed by a much slower elimination phase. It is possible that slower delivery of uranium to the brain allowed most uranium that entered the brain to be sequestered in forms that exchange very slowly.

While it is unlikely that military personnel or civilians will be exposed to uranyl acetate used in this study, uranium that is absorbed following exposure to any form of uranium will be present in the bloodstream as soluble uranium complexes. Previous work has shown that most uranium in the bloodstream is present as the uranyl ion in complexes with various physiological

ligands including hemopexin, complement, and ceruloplasmin (Sutton and Burastero, 2004; Vidaud et al., 2005). The model used in the present work may not account for variations in absorption based on uranium form or route of exposure, but will accurately reflect the distribution and elimination phases of uranium exposure.

Application of stress prior to uranium exposure had little effect on brain or serum uranium levels, though cortical uranium levels were higher 30 days after exposure to 1 mg U/kg in stressed animals. This is consistent with previous findings which demonstrated that moderate stress prior to exposure did not enhance uranium uptake (Barber et al., 2005).

Exposure to a single intramuscular dose of uranium produced adverse effects on locomotion and forelimb grip strength that lasted for at least 30 days. These results are different than those reported by Briner et al. (Briner and Murray, 2005) who observed increased ambulation measured as line crossing in an open field following exposure to uranium in drinking water for 2 weeks or 6 months. Monleau et al. (2005) also reported increased line crossing and rearing in rats exposed to uranium dioxide by inhalation. It is not clear why such divergent effects were observed in this study, however, the duration of exposure may play a role as both of the previous studies utilized repeated exposures to uranium while our study employed a single administration. Application of stress prior to uranium exposure did not alter the effect of uranium on ambulatory activity.

In the present study, we observed decreased forelimb grip strength in rats treated with uranium. Grip strength has been used as a way to measure neuromuscular function for almost 30 years

(Meyer et al., 1979). However, there are a number of other factors that can influence grip strength testing, including sensory impairment and body weight (Maurissen et al., 2003). No lesions of peripheral nerve, sensory ganglia or spinal cord were noted in the animals in this study. Rats exposed to uranium did have lower body weights than the control animals which may have contributed to decreased grip strength. However, rats that were stressed prior to uranium exposure did not exhibit deficits in grip strength. Stressed rats had identical body weights as did unstressed rats suggesting that body weight is not the sole factor influencing grip strength.

Treatment with a single dose of uranium produced transient deficits in passive avoidance which is a measure of working memory. Previous studies with depleted uranium oxide inhalation also demonstrated decreased performance of spatial working memory assessed with a Y-maze (Monleau et al., 2005). Other researchers have reported deficits in memory following one and a half month exposure to 40 mg U/L in drinking water to enriched uranium, but not following similar exposure to depleted uranium (Houpert et al., 2005). Belles et al. (2005) found no effect of DU alone or in combination with stress on passive avoidance following 3 months of exposure via drinking water at up to 40 mg/kg/day. The discrepancy in these results may stem from the use of different dosages, exposure routes and testing paradigms. In the present study, rats were trained prior to application of stress or uranium, so the test does not assess the effects of these treatments on acquisition, but on memory retrieval. Because we also observed reduced locomotion in uranium exposed rats, it is possible that impaired motor function underlies the effect on passive avoidance. However, changes in locomotor activity do not parallel the effects on passive avoidance making it unlikely that they play a significant role.

Application of stress prior to uranium exposure prevented the effect of uranium on passive avoidance. While chronic stress and high levels of corticosterone clearly adversely affects learning an memory, there is evidence that stress of shorter duration or lower magnitude enhances memory performance at times up to 13 days post-stress (Luine et al., 1996). Therefore, the ability of stress to prevent decreased performance in working memory may be due to the application of a moderate stressor that enhanced memory performance.

Unstressed animals treated with 1mg uranium/kg exhibited transient depletion of dopamine in the striatum. Previous work demonstrated that uranium could increase dopamine turnover in striatum (Bussy et al., 2006). No changes in striatal DOPAC levels were observed in the present study, however we did not directly measure dopamine turnover. While there have been no studies on the effect of uranium on dopamine synthesis or dopamine transport, previous work demonstrates that exposure to lead causes a significant decrease in tyrosine hydroxylase activity, the rate limiting step in dopamine synthesis (Kohler et al., 1997), and can trigger release of dopamine (Westerink et al., 2002). Due to chemical similarities of the Pb^{2+} ion and the uranyl (UO_2^{2+}) ion, it is possible that uranium is also affecting these processes. Histological examination of H&E, GFAP, and Fluoro-Jade stained sections of brain, including dopaminergic regions such as substantia nigra and striatum, revealed no changes that would account for this effect. Surprisingly, no changes in striatal dopamine were present in animals that had experienced stress immediately prior to uranium administration. The mechanism underlying this difference is unclear, however, the levels of uranium present in the striatum of stressed and unstressed animals were equivalent, indicating that uranium content of the striatum is not the sole determining factor

in dopamine depletion. While these changes are fairly small, it should be noted that these measurements were performed in five independent treatment blocks with each block containing all treatment groups. This provides a robust analysis and indicates that the changes observed in dopamine are highly reproducible.

In conjunction with the neurological effects observed, all uranium treatments used in this study caused some degree of uremia associated with kidney injury (reported in detail in Zimmerman et al., submitted). Uremia has the potential to cause a number of the adverse effects observed in this study. In previous work using partial nephrectomy to induce mild uremia, mice exhibited reduced weight gain, reduced ambulatory activity, and impaired spatial memory as assessed by a water maze (Al Banchaabouchi et al., 1999). In the present study, the application of stress prior to uranium exposure prevented the adverse effects of uranium on grip strength, passive avoidance, and dopamine levels, but stressed animals had identical levels of BUN and creatinine in serum suggesting that uremia is not the only factor involved. Additionally, neuropathologic alterations associated with uremia were not detected. These consist of peripheral nerve axonal atrophy, secondary demyelination and axonal degeneration, and neuronal degeneration and demyelination in the brain (Dyck et al., 2002; Harper and Butterworth, 2002). Admittedly such changes are features of severe, prolonged uremic states, which was not a feature of our DU intoxication model (Zimmerman et al., submitted).

Most prior reports of the effects of uranium on neurological function have not investigated renal function, despite the use of relatively high doses of uranium for prolonged periods of time. One study does report changes in urinary parameters including a 3-fold increase in urinary gamma

glutamyl transpeptidase and a 2-fold decrease in urinary urea, suggesting tubular damage (Lestaevel et al., 2005). This study identified kidney as a target organ, but did not discuss the potential impact of uremia on their results. A possible role of uremia must be considered when evaluating the neurological effects of uranium due to its nephrotoxicity.

Conclusion: The data in this study demonstrate that a single intramuscular exposure to uranium at doses above 0.3 mg U/kg can cause adverse effects on the nervous system resulting in behavioral, memory, and biochemical changes. Prior application of stress does not exacerbate the effects of uranium on the nervous system and some of the adverse effects of uranium are prevented by prior application of stress. The positive effects of stress are not due to changes in uranium kinetics or distribution, which were unchanged by stress, and are likely due to compensatory changes of the nervous system in response to stress. All uranium treatments produced renal damage with subsequent uremia. Uremia causes adverse effects on nervous system function and may be responsible for some of the adverse effects observed as opposed to a direct effect of uranium. Prior stress did not alter the levels of uremia though they did alter the response to uranium, suggesting that uremia is not solely responsible for the observed effects. Kidney function should be evaluated in all studies dealing with uranium due to the nephrotoxic potential of this metal.

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Table 1. Ambulatory activity in rats exposed to uranium with or without prior stress.

Days after administration	Stress	DU Dose	Ambulatory	Non-ambulatory	Total
6	Non-stressed	0	131.2 ± 8.56	244.2 ± 15.9	374.7 ± 22.65
		0.1	122.3 ± 10.46	265.6 ± 19.49	354.8 ± 23.61
		0.3	99.8 ± 8.59	238.5 ± 15.9	351.0 ± 22.65
		1	88.5 ± 8.54	189.5 ± 15.9	324.5 ± 22.65
	Stressed	0	128.4 ± 8.55	279.6 ± 15.96	388.9 ± 27.74
		0.1	109.7 ± 10.51	227.3 ± 19.65	362.3 ± 27.74
		0.3	106.1 ± 8.62	244.0 ± 15.94	349.5 ± 27.74
		1	98.1 ± 8.56	225.6 ± 15.96	343.5 ± 27.74
13	Non-stressed	0	121.8 ± 8.89	233.4 ± 16.63	339.8 ± 22.65
		0.1	113.8 ± 10.46	247.5 ± 19.49	343.2 ± 22.65
		0.3	102.1 ± 8.59	239.5 ± 15.9	349.0 ± 22.65
		1	97.6 ± 9.07	213.5 ± 17.07	330.2 ± 22.65
	Stressed	0	117.2 ± 8.7	247.0 ± 16.3	277.5 ± 22.64
		0.1	106.5 ± 10.51	228.3 ± 19.65	310.3 ± 24.18
		0.3	107.8 ± 8.77	267.5 ± 16.27	334.1 ± 22.64
		1	98.5 ± 8.71	242.9 ± 16.3	297.3 ± 22.64
20	Non-stressed	0	124.6 ± 8.56	227.0 ± 15.9	408.8 ± 22.73
		0.1	102.5 ± 10.46	246.0 ± 19.49	364.9 ± 23.18
		0.3	103.9 ± 8.59	243.6 ± 15.9	365.7 ± 22.73
		1	105.6 ± 8.54	229.0 ± 15.9	359.9 ± 22.73
	Stressed	0	108.6 ± 8.55	256.3 ± 15.96	335.9 ± 28.03
		0.1	107.0 ± 10.51	211.2 ± 19.65	333.7 ± 28.03
		0.3	108.7 ± 8.62	254.0 ± 15.94	317.1 ± 28.03

		1	100.7 ± 8.56	231.1 ± 15.96	325.4 ± 28.03
27	Non-stressed	0	109.6 ± 8.56	215.6 ± 15.9	349.6 ± 22.78
		0.1	108.5 ± 10.46	234.0 ± 19.49	375.0 ± 23.22
		0.3	93.9 ± 8.59	234.7 ± 15.9	362.2 ± 22.78
		1	93.0 ± 8.54	204.9 ± 15.9	328.4 ± 22.78
	Stressed	0	109.7 ± 8.55	249.4 ± 15.96	323.1 ± 22.75
		0.1	97.3 ± 10.51	229.1 ± 19.65	341.0 ± 23.19
		0.3	106.6 ± 8.62	222.3 ± 15.94	331.2 ± 22.75
		1	90.5 ± 8.56	228.0 ± 15.96	317.9 ± 22.75

Table 2. Body weights of rats exposed to uranium with or without prior stress. Values are mean ± SD (N=10-15). * indicates values that are significantly different from saline only controls ($p<0.05$).

Day post-dosing	Uranium dose (mg/kg)	Body Weight (g)	
		Unstressed	Stressed
-1	0	321.6 ± 16.1	325.6 ± 15.4
	0.1	321.7 ± 14.3	323.9 ± 13.0
	0.3	323.5 ± 11.6	318.61 ± 12.0
	1.0	322.5 ± 14.0	321.0 ± 11.9
6	0	352.8 ± 18.2	359.0 ± 16.8
	0.1	339.9 ± 20.7	336.4 ± 16.9
	0.3	329.5 ± 17.7*	322.1 ± 19.5*
	1.0	317.8 ± 17.3*	315.4 ± 15.4*
13	0	365.3 ± 20.1	376.9 ± 17.8
	0.1	357.7 ± 26.8	367.3 ± 21.7
	0.3	350.4 ± 17.0	344.8 ± 22.3*
	1.0	339.1 ± 20.1*	341.5 ± 18.0*
20	0	377.8 ± 25.1	391.5 ± 17.8
	0.1	373.6 ± 32.2	382.9 ± 23.6
	0.3	371.8 ± 23.3	363.8 ± 26.4
	1.0	362.7 ± 24.1	363.9 ± 20.9
27	0	389.9 ± 22.2	402.4 ± 20.8

	0.1	382.8 ± 34.2	395.4 ± 27.7
	0.3	387.0 ± 25.3	378.8 ± 25.9
	1.0	379.0 ± 28.2	379.8 ± 21.9

Table 3. Grip strength in rats exposed to uranium with and without prior stress. Values are mean ± SD (N=10-15). * indicates values that are significantly different than saline only controls ($p<0.05$).

Day post-dosing	Uranium dose (mg/kg)	Forelimb Grip Strength (kg)		Hindlimb Grip Strength (kg)	
		Unstressed	Stressed	Unstressed	Stressed
6	0	1.83 ± 0.04	1.81 ± 0.05	0.94 ± 0.04	0.97 ± 0.03
	0.1	1.87 ± 0.04	1.79 ± 0.05	0.85 ± 0.05	1.02 ± 0.07
	0.3	1.79 ± 0.03	1.76 ± 0.03	0.88 ± 0.04	0.91 ± 0.04
	1.0	1.70 ± 0.07*	1.79 ± 0.04	0.89 ± 0.04	0.89 ± 0.04
13	0	1.96 ± 0.04	1.94 ± 0.04	0.95 ± 0.05	1.03 ± 0.04
	0.1	2.01 ± 0.02	2.05 ± 0.03	0.93 ± 0.06	1.07 ± 0.04
	0.3	1.91 ± 0.04	1.96 ± 0.04	0.95 ± 0.03	0.97 ± 0.05
	1.0	1.86 ± 0.04*	1.93 ± 0.03	0.92 ± 0.04	0.98 ± 0.03
20	0	2.01 ± 0.06	1.98 ± 0.05	0.97 ± 0.05	0.98 ± 0.04
	0.1	1.98 ± 0.07	2.06 ± 0.04	0.96 ± 0.04	1.09 ± 0.03
	0.3	1.90 ± 0.04	1.94 ± 0.05	0.99 ± 0.04	0.98 ± 0.06
	1.0	1.87 ± 0.06*	1.96 ± 0.04	0.93 ± 0.04	0.98 ± 0.03
27	0	2.06 ± 0.06	2.08 ± 0.05	1.00 ± 0.06	1.06 ± 0.04
	0.1	2.08 ± 0.03	2.09 ± 0.07	1.06 ± 0.05	1.15 ± 0.04
	0.3	1.99 ± 0.04	2.10 ± 0.04	0.97 ± 0.03	1.08 ± 0.07
	1.0	1.91 ± 0.05*	1.99 ± 0.04	1.00 ± 0.05	1.00 ±0.05

Table 4. Effect of treatments on renal function.

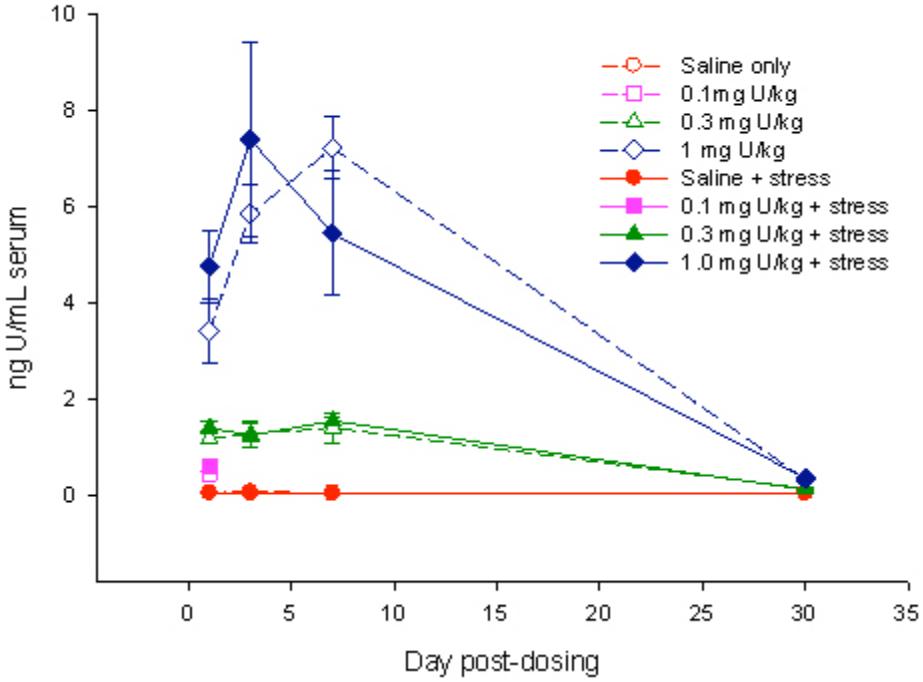
Serum Urea Nitrogen (mg/dl)

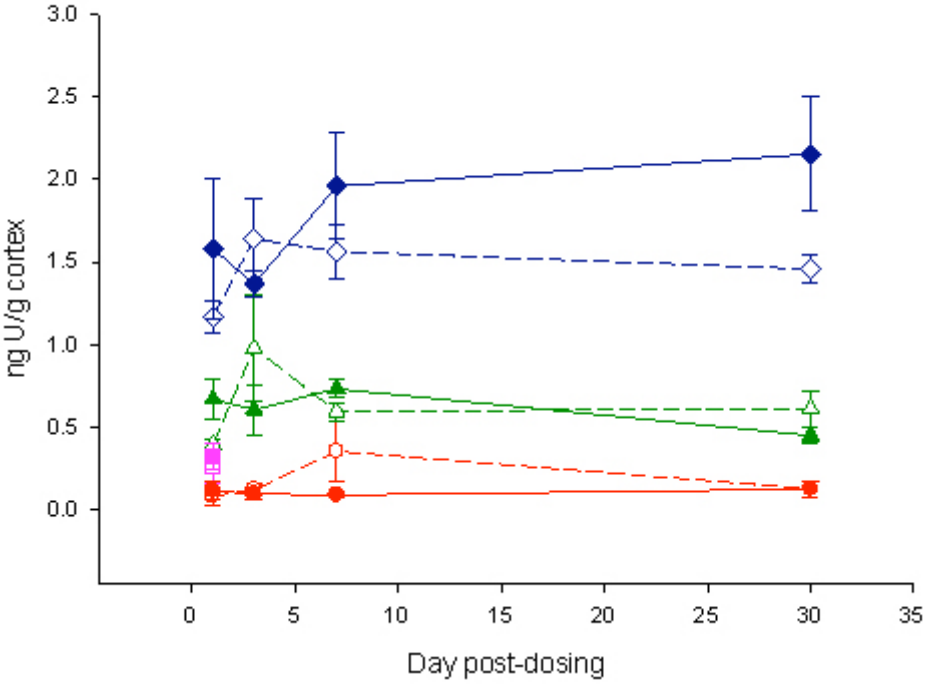
Treatment Group	Day 1	Day 3	Day 7	Day 30
1	22.0 ± 3.3	22.6 ± 1.5	23.2 ± 3.4	25.8 ± 3.6
2	22.2 ± 0.8	35.7 ± 6.2*	25.6 ± 4.7	26.2 ± 5.0
3	24.4 ± 3.4	46.0 ± 3.5*	55.2 ± 36.9	23.2 ± 1.1
4	24.2 ± 4.9	56.0 ± 6.0*	104.8 ± 29.1*	27.0 ± 2.6
5	22.0 ± 2.6	23.8 ± 1.1	23.4 ± 1.8	22.6 ± 1.1
6	23.3 ± 2.1	36.8 ± 4.0*	27.2 ± 1.6	23.6 ± 1.3
7	23.7 ± 3.4	50.2 ± 5.0*	62.2 ± 21.9	26.6 ±3.6
8	25.0 ± 1.4	61.0 ± 11.6*	110.0 ±44.5*	26.8 ± 2.4

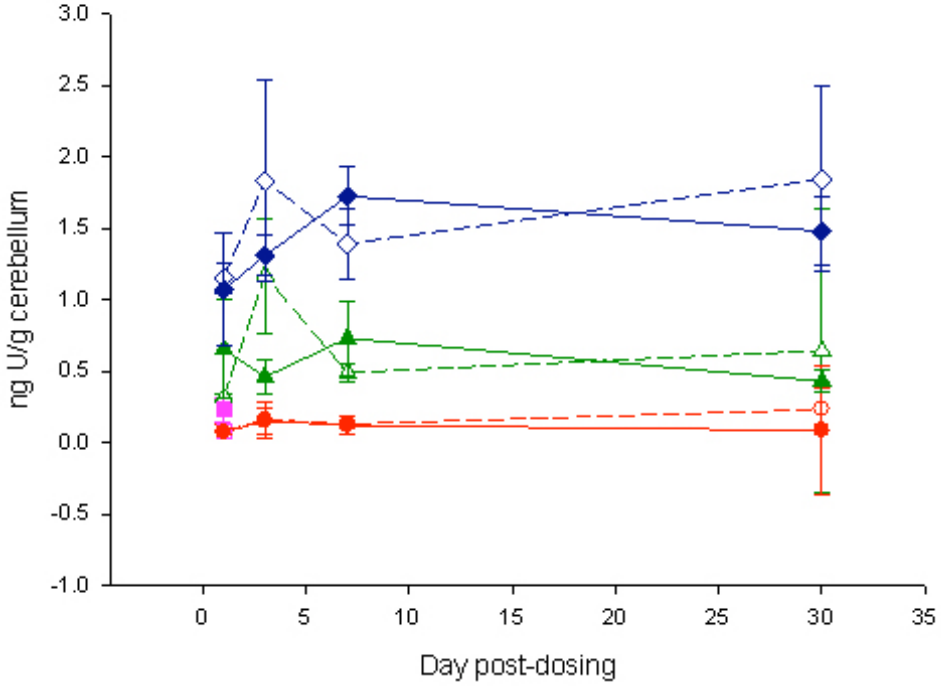
Values are mean ± SD (N=10-15) *indicates values that are significantly different from control (Group 1), ($p < 0.05$)

Treatment Groups: 1- saline control; 2- 0.1 mg/kg DU; 3- 0.3 mg/kg DU; 4- 1.0 mg/kg DU;

5- stress; 6- stress + 0.1 mg/kg DU; 7- stress + 0.3 mg/kg DU; 8- stress + 1.0 mg/kg DU


Saline only
0.1mg U/kg
0.3 mg U/kg
1 mg U/kg
Saline + stress
0.1 mg U/kg + stress
0.3 mg U/kg + stress
1.0 mg U/kg + stress
ng U/mL serum
Day post-dosing


ng U/g cortex
Day post-dosing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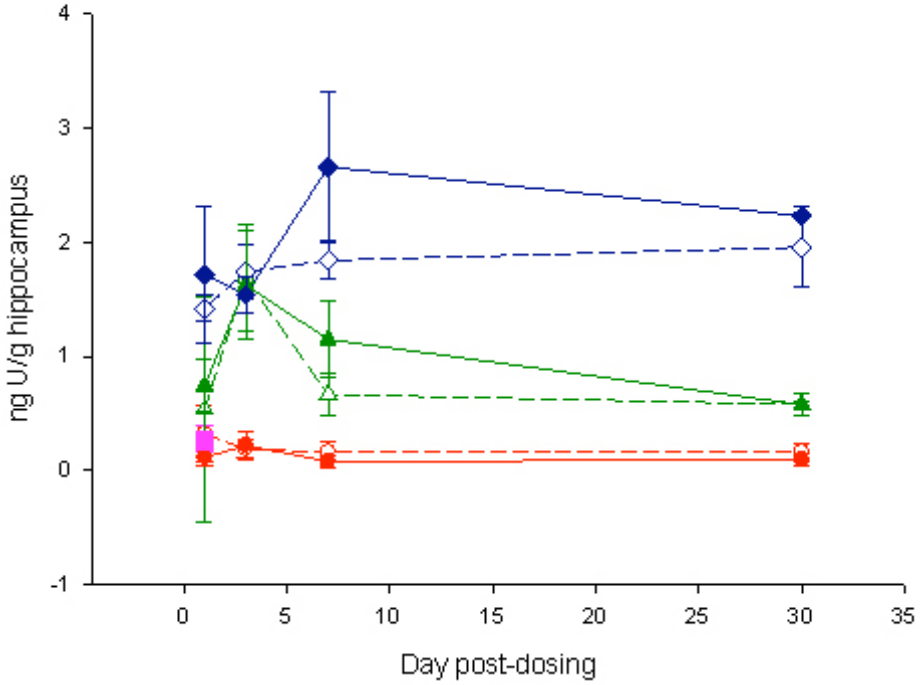
Day post-dosing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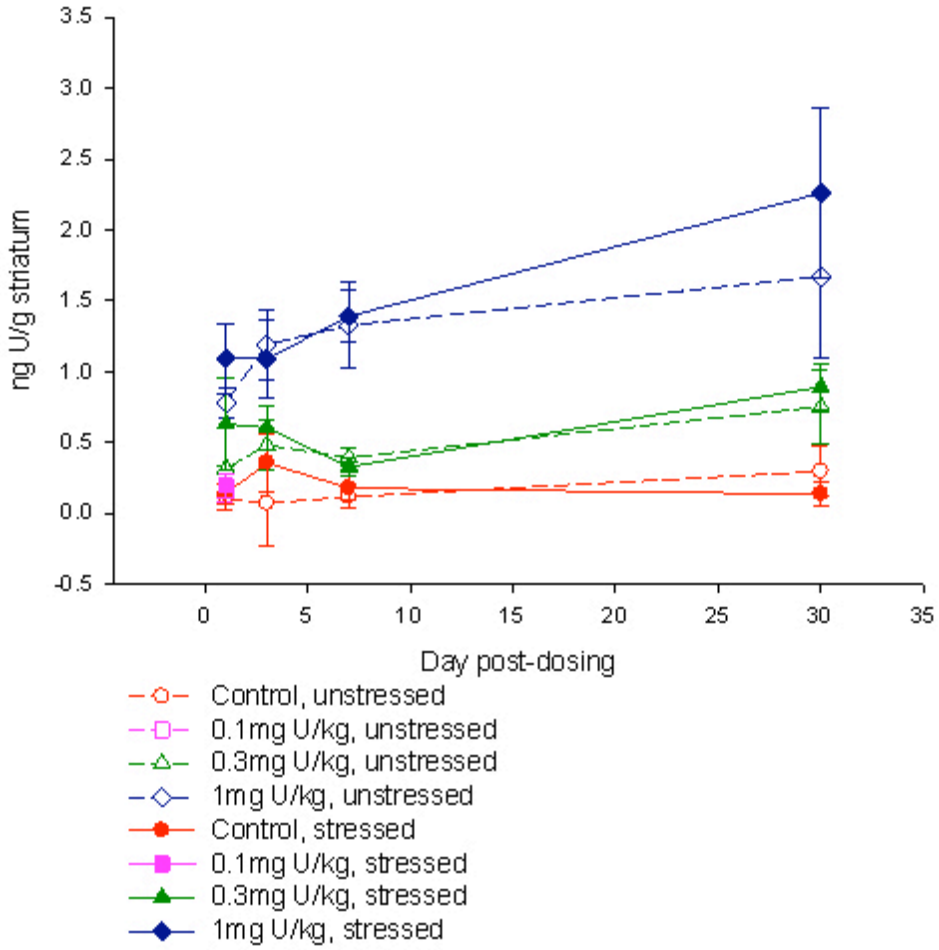

Figure 1. Uranium concentration of serum and brain regions from rats treated with uranium. A- serum, B- cerebral cortex, C- cerebellum, D- hippocampus, and E- striatum. Values are mean ± SD (N=4-5).

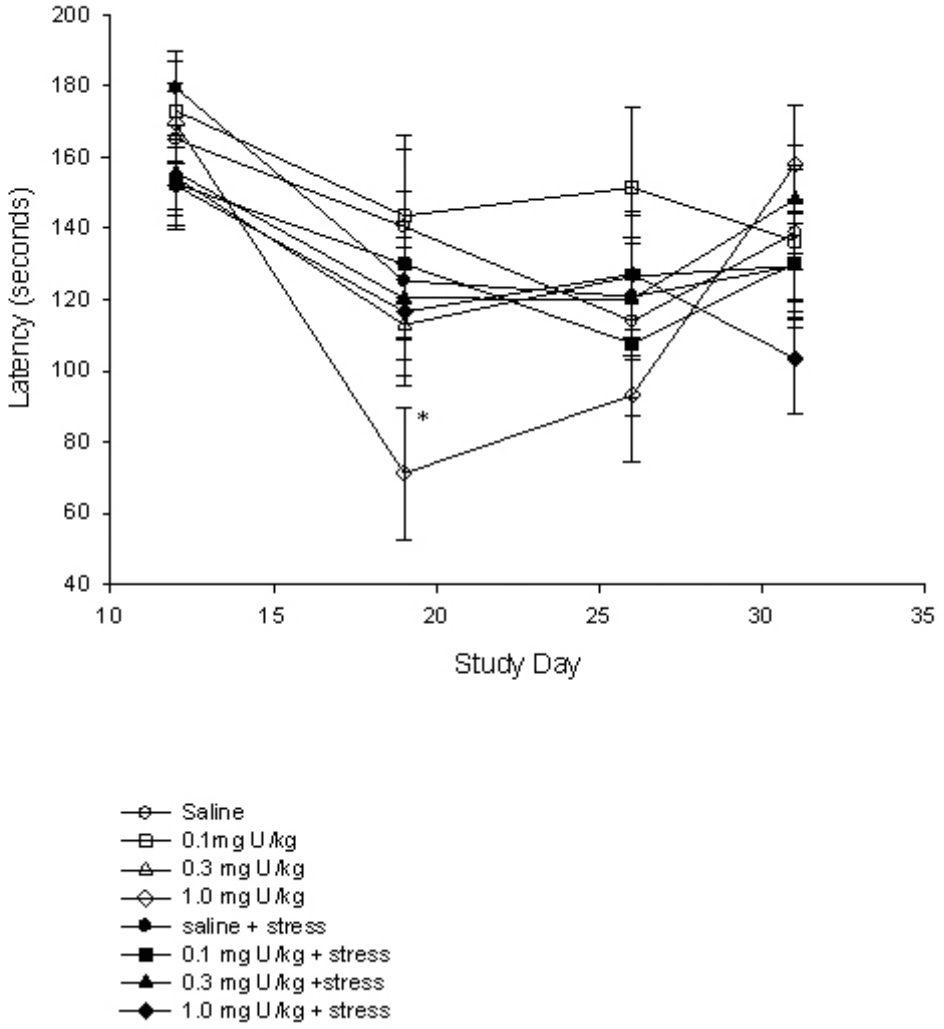

Figure 2. Latency times in passive avoidance assay. Values are mean ± SEM (N=10-15). * indicates values that are significantly different from saline only ($p<0.05$).

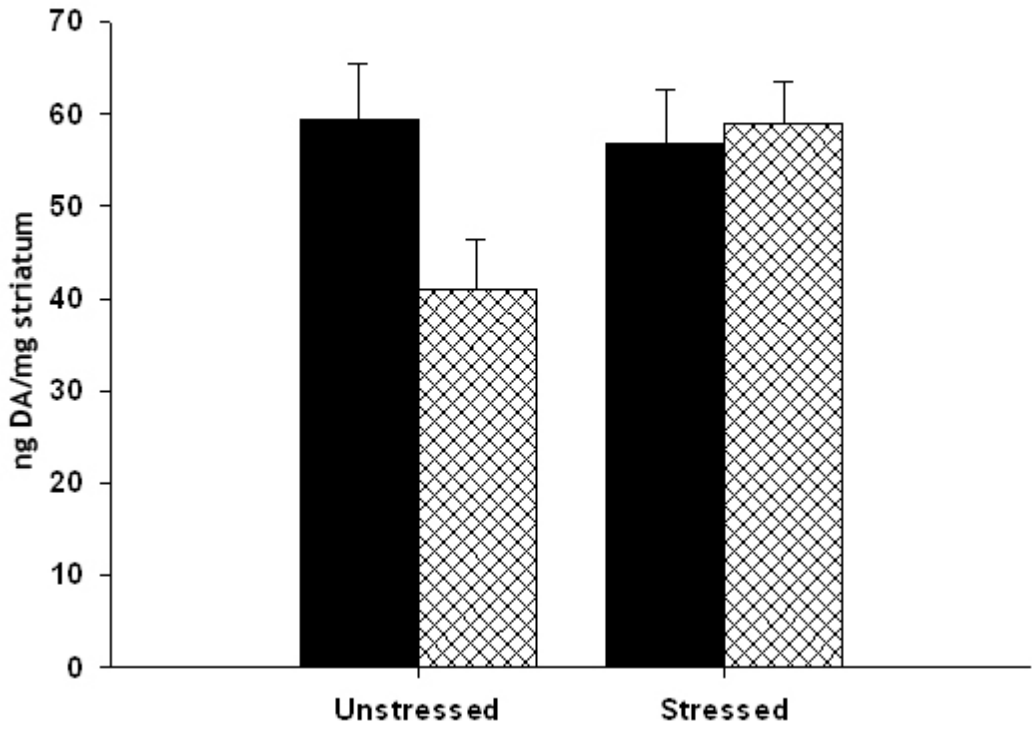

Figure 3. Striatal dopamine content 3 days after exposure to 1 mg U/kg. Values are mean ± SD (N=5). * indicates values that are significantly different from control ($p<0.05$).

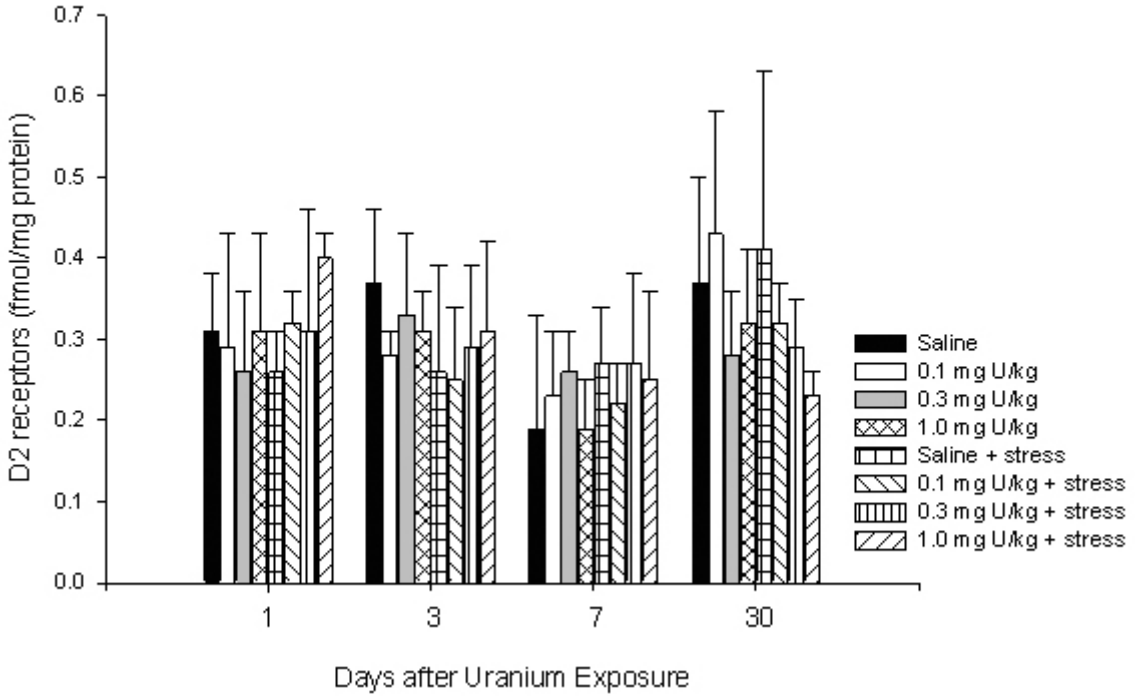

Figure 4. Striatal D2 dopamine receptor numbers following exposure to uranium with or without stress. Values are mean ± SD (N=5). No significant treatment related effects were observed at any timepoint.

Supplemental Data

Table A1. P-values from analysis of FOB categorical response variables.
(note: No analysis is presented for response variables that did not change throughout the study; these include in cage posture, clonic and tonic movements, vocalizations, respirations, activity level; handling cage removal ease, lacrimation, salivation, piloerection, palpebral closure and reflex, oculocardiac response, pupil size and light response, visual placing, open field clonic and tonic movements, fecal, gait, gait score, mobility score, stereotypy, righting and tail-limb reflexes)

		Days after administration of DU			
Response	**Effect**	**6**	**13**	**20**	**27**
Handling: reactivity	Stress	0.7739	0.6392	0.9688	0.6546
	DU	0.4793	0.8964	0.4393	0.6612
	Stress*DU	0.9468	0.1868	0.6010	0.5198
Open field: arousal	Stress	0.0662	0.6996	0.9321	0.6423
	DU	0.3152	0.4776	0.1643	0.5771
	Stress*DU	0.4552	0.1676	**0.0423**	0.2750
Open field: rearing	Stress	0.1185	0.1907	0.1822	0.7164
	DU	0.4308	0.3088	0.6527	0.1744
	Stress*DU	0.6297	0.9005	0.3720	0.4365
Open field: urine pools	Stress	0.0922	0.6359	-	-
	DU	0.9811	0.5724	-	-
	Stress*DU	0.8098	0.6580	-	-
Open field: bizarre behavior	Stress	-	-	0.3129	0.9440
	DU	-	-	0.9641	0.1425
	Stress*DU	-	-	0.8283	0.6114
Approach response	Stress	0.3319	0.3502	0.9982	0.8648
	DU	0.6354	0.1898	0.1131	0.9570
	Stress*DU	0.6969	0.8191	0.1279	0.9243
Touch response	Stress	0.5493	0.9565	0.6931	0.7858
	DU	0.4197	0.1376	0.8932	0.4391
	Stress*DU	0.2520	0.2496	0.8676	0.6236
Click response	Stress	0.9543	0.0140	0.8755	0.7472
	DU	0.4077	0.4326	0.9762	**0.0452**
	Stress*DU	0.5749	0.0924	0.3478	0.2265
Pinch response	Stress	0.2821	0.8653	0.6783	0.6332
	DU	0.2959	0.7342	0.1189	0.6996
	Stress*DU	0.2226	**0.0148**	0.2292	**0.0310**
Rotarod agility score	Stress	0.3162	0.4691	0.8799	0.5638
	DU	0.6897	0.6687	0.6703	0.4080
	Stress*DU	0.9880	0.8796	0.4716	0.1655

TEMPORAL CLINICAL CHEMISTRY AND MICROSCOPIC RENAL EFFECTS FOLLOWING ACUTE URANYL ACETATE EXPOSURE.

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Military use of depleted uranium (DU) has renewed interest in the toxicology of this metal. We assessed the nephrotoxicity of single exposure DU with and without pre-exposure stress. Adult male Sprague-Dawley rats (n=418) were administered a single im dose of 0, 0.1, 0.3 or 1.0 mg/kg DU. Just prior to day 0 dosing, rats were exposed to swimming stress resulting in plasma corticosterone concentrations (ng/ml, mean +/-SD) 763.65+/-130.94 and 189.80+/-90.81 for stressed and unstressed rats respectively. Serum and renal tissue for uranium concentration, hematocrit, serum chemistry profile, and renal histology were assessed on sacrifice days 1, 3, 7 and 30 (n=5/ group/day) post-DU-dosing. Dose related increases in serum and tissue uranium values were noted. In the interval assessed, DU concentration peaked on day 1 in the kidney and day 7 in the serum. Statistically significant dose-related elevations of serum creatinine (Cr) and blood urea nitrogen (BUN) concentrations were seen on days 3 and 7. A noteworthy decline in serum albumin coincided with Cr and BUN changes suggesting a protein losing nephropathy. Lesions included dose related acute tubular necrosis and proliferative glomulonephritis. Tubular regeneration occurred rapidly in low dose (0.1 mg/kg) rats and was almost complete by day 30. High dose rats had more extensive tubular necrosis and a delayed regenerative response, with regions of focal residual chronic interstitial nephritis and cortical scarring on day 30. Glomular changes were reversed in all treatment groups by day 30. Stress exposure had no impact on any measured parameter other than plasma corticosterone.

Injury to the proximal renal tubular epithelium occurs with low level dosing of depleted uranium (DU) and renal concentrations of ≥ 3 μg uranium/gram of tissue (Diamond et al. 1989). Cessation of exposure led to diminished renal uranium concentrations and associated regeneration of tubular epithelium. Modulation of this toxic effect by stress has not been previously evaluated. We present sequential correlative evaluations of renal uranium concentrations, tubular injury and clinical chemistry determinations to document the progression/recovery of the nephropathy. Following a single exposure to DU in rats our work includes and an assessment of whether a well-documented pre-dosing stress modulates renal uranium distribution, lesions, and function. The research reported here is part of a larger project evaluating the neurotoxicity of depleted uranium exposure in rats and its modulation by stress (Barber et al. 2006).

Depleted uranium is a by-product of the enrichment process for that metal, during which the more radioactive isotopes U^{235} and U^{234} are removed (Pellmar et al. 1999; Squibb et al. 2005). This depleted form of uranium has about 60% of the radioactivity of the natural mineral, and its density availability and relatively low cost make it attractive for military purposes, specifically in armor and projectiles (McDiarmid et al. 2000). This use has resulted in exposures to the metal by aerosol, ingestion and wound contamination. In the present work we report nephrotoxic effects following exposure to various dosages of soluble depleted uranium with and without pre-dosing stress.

Stress may be defined as the imbalance between environmental demands for survival and the individual's capacity to adapt (Marshall et al. 2000; Lazarus and Folkman 1984). Individuals react to stress by shifting resources from other biological activities (such as reproduction or growth) toward survival. The degree of these responses relates to the intensity and duration of the stress. Milder forms of stress draw on reserve resources, but severe acute or chronic stress may impact on critical metabolic pathways, which are reflected in endocrine, behavioral, autonomic and immunological events. Thus, severe acute or chronic stress episodes may negatively alter homeostatic metabolic events, diminishing ability to resist infectious agents or tolerate toxicant exposures. As regards the latter, stress may have varying effects on toxicity. Ehrich et al. (1986) demonstrated both amelioration and enhancement of organophosphate-induced delayed neuropathy associated with elevated concentrations of stress-related hormones. However, few alterations of organophosphate-induced effects were noted in stressed animals after a single toxicant exposure at a dose that caused no clinical evidence of poisoning (Pung et al., 2006).

There are few studies on the effects of stress on heavy metal toxicity. Cory-Slechta et al. (2004) demonstrated that restraint stress in pregnant rats potentiated the toxic effects of lead in the offspring. Relative to modulation of experimental kidney disease, heat stress applied six (but not 48) hours prior to ischemic insult protected against acute renal injury (Kelly 2002). This was thought to be related to the actions of induced heat shock proteins. However, these proteins were thought not to be a factor in protection against uranium-induced nephropathy (Tolson et al. 2005). In the present study, pre-dosing forced swimming was employed to determine if such stress affected temporal nephrotoxic effects of depleted uranium as demonstrated by evaluation of uranium serum and renal tissue concentrations, renal lesions, and clinical chemistry data.

METHODS

Male Sprague-Dawley rats (n=418) with a starting weight of 250 to 300 grams were used in this study. The experiment was conducted in a two by four randomized complete block

design, with two levels of stress (yes/no) and four levels of DU exposure (0.0, 0.1, 0.3, 1.0 mg/kg). There were five blocks. On sacrifice days one, three, seven, and 30, three cohorts were created. One cohort (n=5/dose/day) was used for renal pathology and clinical biochemistry. The second cohort of similar size was used for analysis of blood and kidney uranium concentrations, and in part for renal histopathology. A third cohort was perfusion-fixed for detailed neuropathology. The latter was reported elsewhere (Barber et al. 2006) and is not of this report.

The rats were singly caged in a climate controlled facility, at 21-22 degrees C with a daily12 hour light-dark cycle. They were subject to daily stress for five days prior to dosing with depleted uranium. This included restraint on days one through four and swimming on day five. For restraint stress, each animal was placed in a Plexi-glas™ tube (six centimeters diameter by 22 centimeters long) (Konarska et al. 1989) with adequate breathing holes for a 20-minute period and then returned to its home cage. For swim stress, each animal was placed in a tank of water at 23-25 degrees C and allowed to swim for a 30-minute period. When swimming was complete, the animal was towel-dried for one-two minutes and placed under a warming lamp for an additional two-three minutes. Animals in the no stress groups were handled daily by removing them from the home cage, placing them in a box and immediately returning them to their home cage.

Within ten minutes after day five swim stress or handling (no-stress groups), rats were anesthetized with isofluorane. Whole blood was collected from the orbital sinus into heparinized microcentrifuge tubes (Becton-Dickinson, Lincoln Park, NJ). Plasma, for corticosterone measurement, was separated from whole blood by centrifugation at 12,000 rpm at four degrees C for four minutes. Separated plasma was frozen at -70 degrees C until analysis using a corticosterone ^{125}I-radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA). Following blood collection, rats were intramuscularly dosed with DU (uranyl acetate) prepared in saline at one of four dosage concentrations (0.0, 0.1, 0.3, or 1.0 mg/kg) dependent upon their group assignment. The day of DU exposure was considered day 0 of the study.

Animals were randomly selected prior to the study onset from each group for sacrifice by CO_2 euthanasia on post-dosing days one, three, seven and 30. At sacrifice, blood was collected from the inferior vena cava into serum separator and sodium heparin tubes (Becton-Dickinson, Franklin Lakes, NJ). The serum separator tube was used for serum uranium concentration and serum biochemistry value measurements. For dosage groups two and six [0.1 mg/kg, no stress and stress] serum uranium concentrations were only measured on sample day one. For all groups on all sample days, serum sodium (Na^+), potassium (K^+), chloride (Cl^-), calcium (Ca^{++}), total carbon dioxide ($TC0_2$), glucose (Glu), blood urea nitrogen (BUN), creatinine (Cr), phosphorus (P), total protein (TP), albumin (Alb), globulin (Glb-calculated), total bilirubin (TBil) values, and alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities were measured. Hematocrit percentage was determined using heparinized whole blood placed in a microcentrifuge tube (Becton-Dickinson, Lincoln Park, NJ) and centrifugation (Hematastat, Separation Technology Inc., Altamonte Springs, Florida). Kidney tissue was collected and frozen at -70 degrees C for uranium analyses. In addition, a complete transverse section was immersion-fixed in 10% neutral buffered formalin for histological study. The Virginia Tech Institutional Animal Use and Care Committee (Blacksburg, VA) approved protocols and procedures used in the study.

Serum biochemistry samples were measured within one hour of sample collection using an Olympus AU400 analyzer (Olympus, Center Valley, PA) by the Virginia-Maryland Regional College of Veterinary Medicine Clinical Pathology Laboratory (Blacksburg, VA). Statistical

analysis of the results for each individual analyte was done using commercially available software (Minitab 14, Minitab Inc., State College, PA). A general linear model was used for univariate analysis of variance of mean analytes values. The regression model consisted of the following main effects: stress exposure (y/n), dose (0.0, 0.1, 0.3, and 1.0 mg/kg), sample day (1, 3, 7, and 30) and the following interaction effects: stress-dose, stress-day, dose-day, and stress-dose-day. Effects deemed significant were those with p-values less than or equal to an α-level of 0.05.

Serum and kidney uranium concentration analyses were performed by inductively coupled plasma-mass spectrometry (ICP-MS) on an HP 7500a ICP-MS (Agilent Technologies, Santa Clara, CA) preformed by the Analytical Section of the Hazard Identification Core in the Southwest Hazardous Waste Program (Tucson, AZ). Samples were placed in 15 ml sealed glass pressure tubes with 0.5 ml of concentrated metal-free nitric acid (Optima, Fisher Scientific). Samples were heated to 140 degrees C for two hours in a silicone oil bath, then 0.5 ml of 30% hydrogen peroxide (Ultrex II, J.T. Baker, Phillipsburg, NJ) was added and samples heated at 110 degrees C for an additional 60 minutes. Samples were cooled, quantitatively transferred to acid-washed 5ml volumetric flasks, and brought to volume with deionized water (ElgaStat Maxima, Elga, High Wycombe, UK). ICP-MS analysis on the HP 7500a was conducted using iridium as an internal standard. Uranium concentration was determined from a standard curve of uranium based on the m/z 238 signal. Five repetitions were performed per sample and the average used to calculate uranium concentration. The limit of quantitation of this method was 0.002 ppb. Recovery was determined from samples spiked with 0.1-10 ppb uranium and determined to be 96-108%. The limit of quantitation of this method under normal conditions was 0.01 ppb. By using a lower range of standards, the limit of quantitation was improved to 0.002 ppb to accurately determine uranium concentrations from samples with low levels of uranium. Statistical analyses for serum and tissue uranium concentration were done as previously described for serum chemistry analytes.

For histopathology, formalin fixed kidney tissue was embedded in paraffin, sectioned at six μm thickness and stained with hematoxylin and eosin (H&E). The renal proximal tubular lesions were qualitatively evaluated. In addition, a blinded assessment was made of changes in the outer medullary stripe and adjacent deep cortex of the six μm thick H&E stained sections, using the following grading system: grade 0- no lesions (Figure 1A); grade 1- focal injury of tubular epithelium manifest by cytoplasmic vacuolization and occasional necrotic/apoptotic cells (Figure 1B); grade 2- diffuse necrosis of tubular epithelium (Figure 1C); grade 3- diffuse necrosis with focal early tubular regeneration (Figure 1D); grade 4- extensive early tubular regeneration (Figure 1E); grade 5- advanced regeneration (Figure 1F). Selected sections were stained with terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) procedures. Control rat kidney sections treated with DNAse served as positive controls. The commercial TUNEL assay (Boehringer Mannheim, GmbH Mannheim, Germany) identifies apoptotic cells by using terminal deoxynucleotidyl transferase (TdT) to transfer fluorescein-dUTP to these strand breaks of cleaved DNA. In addition, sections from selected blocks were cut at three μm thickness and stained with the H&E and Masson's trichrome procedures for detailed study of glomerular lesions.

RESULTS

Corticosterone Concentration

Plasma corticosterone concentration was 189.8 ng/ml with a standard deviation (SD) of 90.81 in the non-stressed rats (Groups 1-4). Plasma corticosterone concentration of stressed groups 5-8 was 763.6 with a SD of 130.94 (n=206 stressed, 200 not stressed). These values were significantly different ($p<0.05$).

Uranium Concentration

Mean serum uranium concentrations are shown in Table 1. There was no effect of pre-dosing stress on serum uranium concentrations ($p>0.05$, Figure 2A, Table 1). However, individual main effects due to dose, day, and a dose-day interaction effect on the mean serum uranium concentration were seen (Figures 2A, 2B, Table 1). Serum uranium concentrations were elevated in rats administered depleted uranium, in particular those given the 1.0 mg/kg dose (Table 1). This elevation was marked one day post-dosing with further increase seen on day seven and return to control values by day 30.

Mean kidney tissue uranium concentrations are shown in Table 1. Uranium was concentrated in the renal tissue, being present in μg U/gram of tissue quantities in contrast to the ng U/ml seen in the serum. There was no effect of pre-dosing stress on tissue DU concentrations ($p>0.05$, Figure 3A, Table 1). As with serum uranium concentration, individual main effects due to dose and day, and the dose-day interaction effect on the tissue uranium concentration were seen (Figures 3A, 3B) Renal concentration increased rapidly in all dosage groups receiving DU in a dose dependent fashion (Figures 3, 4, Table 1). Unlike serum uranium concentrations, values were highest on day one then declined throughout the sample period (Figure 5).

Clinical Pathology

Table 2 shows serum chemistry analytes which demonstrated significant ($p<0.05$) mean analyte value differences associated with main and interaction effects. Mean and SD values for serum chemistry analytes with significant effect differences are shown in Table 3. Of the statistically different means, the following were considered to be of clinical and toxicological significance: BUN (dose, day, dose-day [Figure 6]), Cr (dose, day, dose-day [Figure 7]), and Alb (dose, day [Figure 8]). Stress had no impact on analyte response patterns across dosage groups or across sample days. Serum Cr and BUN values were elevated in comparison to control groups one and five (0.0 mg/kg DU) in a dose-day interaction effect for dosage groups three (0.3 mg/kg [two fold increase]) and four (1.0 mg/kg [four fold increase]) beginning on day 3, peaking on day 7, and returning to control values by day 30 (Figures 6B, 7B). Serum albumin mean values varied significantly with separate main effects of day and dosage (Figure 8A). Again variance suggested a dose dependent relationship with the most noteworthy decline in albumin concentration occurring in the high dosage groups four and eight (1.0 mg/kg) as shown in Figure 8A. Inversely and simultaneously to the increases seen with BUN and Cr, albumin declined on day three with a maximum decline on day seven and a return to normal on day 30 (Figure 8A) . While not statistically significant ($p=0.167$), the interaction effect of dose-day on mean serum albumin value followed an inverse pattern to that seen with BUN and Cr with the highest dosage group demonstrating the most noteworthy decline on day seven (Figure 8B). Changes in these analyte results were preceded by increases in kidney uranium concentrations (Figures 4, 5, Table 1).

Renal Histopathology

Lesions were most prominent in the epithelial cells lining the proximal tubules. Early injury was manifest by loss of the brush borders as seen with the PAS stain. Examination of the H&E stained sections revealed a sequence of change, as indicated in the lesion scoring scale (see Methods). Compared to controls (Figure 1A), there was cytoplasmic swelling and vacuolization, and the presence of occasional necrotic or apoptotic cells (grade 1, Figure 1B). This was followed by expansion of necrosis to involve large numbers of tubular epithelial cells, with sloughing of cellular debris into the lumen (grade 2, Figure 1C). Subsequently, focal regions of flattened basophilic regenerating cells appeared in the walls of the tubules, displacing necrotic cells (grade 3, Figure 1D), initially noted in the outer medulla. These regenerating cells became more prominent, occupying large regions of the affected tubular surface (grade 4, Figure 1E). With time the regenerating epithelium matured to resemble control cells (grade 5, Figure 1F), although there was some residual focal scarring and interstitial inflammation in the high dose rats. These proximal tubular changes primarily involved the outer stripe of the medulla, adjacent deep cortex and the medullary rays (Figure 9). TUNEL staining revealed only occasional positive nuclei in contracted tubular epithelial cell during periods of active injury. This was absent in other dying cells. The DNAse-treated (positive control) sections had prominent TUNEL-positive nuclei.

Assessing the proximal tubular lesions with the scoring scale demonstrated progression was related to dosage and post-exposure day. Stress was not a factor. The earliest stage of injury (grade 1) was frequent on post-dosing day one in the 0.3 and 1.0 mg/kg dose groups, but was occasionally seen in the 0.1 mg/kg group rats (Figure 1B). There was further dose dependent differentiation in lesion progression in tissues studied the third post-dosing day. The 0.1 mg/kg animals had largely progressed to grade 3, in which extensive tubular epithelial necrosis was seen, with associated early regeneration (stress > no stress) (Figure 1D). The 1.0 mg/kg rats were mostly restricted to grade 2, where diffuse necrosis was seen without regeneration (Figure 1C). The mid-dose animals were intermediate in these day three pathological responses. By post-dosing day seven, all dosages were in the grade 4 stage, indicating extensive active regeneration, and on day 30 this process was largely completed (Figures 1E, 1F).

Glomeruli from 0.3 and 1.0 mg/kg DU dose groups had focal, segmental of varying degree, mesangial expansion and hypercellularity, consistent with mesangial proliferation (Brun and Olsen, 1981), plus the presence of red-stained (by Masson's trichrome) droplets in this region (Figure 10). This was noted on post-dosing days three and seven, but was absent on day 30.

DISCUSSION

Uranium, as other heavy metals, is a well-known nephrotoxin, accumulating in the kidney and injuring proximal tubular epithelial cells, but preserving the basal lamina (Lim et al. 1987). Following absorption the metal is carried in the blood plasma as uranium-transferrin or in low molecular weight anionic complexes. Some 80% of this uranium is filtered by the glomerulus. It subsequently binds to anionic sites in the proximal tubular epithelial brush border in the form of UO_2^{++}, and enters the cell by endocytosis (Leggett 1989). Injury to the cell is related to alterations in cell and lysosomal membranes, injured mitochondria leading to impaired energy production and altered calcium ion homeostasis (Leggett 1989). These may be based upon uranium-related induction of cellular oxidative stress (Tanlan et al. 2004).

We report that a single intramuscular exposure to 0.1, 0.3 or 1.0 mg/kg of soluble depleted uranium to male Sprague-Dawley rats, given as uranyl acetate, leads to transient prominent necrosis of proximal tubular epithelium, with associated dose-related elevations of

renal uranium concentration and plasma creatinine and BUN. The necrotic lesions evolve into widespread early regeneration in the seven-day post-dosing period. There is dose-related delay in the initiation of regeneration, as the latter is seen earliest in the low-dose group. However, the mid-and high-dose groups "catch up" as renal uranium levels are reduced. Regeneration is largely complete by day 30 in all exposure levels.

The significant difference in plasma corticosterone concentration between groups exposed to the stress event and those not provides convincing evidence that stress difference existed between various animals. Since dosing with depleted uranium was done shortly after blood sample collection, it is likely that the toxic exposure was achieved under conditions of markedly elevated plasma corticosterone for the stressed rats. We found no evidence that stress affected renal uranium concentrations, or the nature, severity and progression of the uranium-induced tubular injury.

There have been other experimental animal studies of soluble depleted uranium nephrotoxicity. Depleted uranium salts have been administered using single or multiple exposures (Diamond et al. 1989; Haley et al. 1982; Kato et al. 1994; Lim et al. 1987). The injury in these appeared to be due to heavy metal toxicity rather than radiation effects. Pertinent to our work are reports of single exposure studies. Haley et al. (1982) used a single subcutaneous exposure of 10 mg/kg uranyl nitrate to rats. Although their dose was much higher (10 times our highest exposure, administered subcutaneously rather than by our intramuscular route), they noted similar changes to ours, such as proximal tubular injury over a 5 day post-dosing period, with subsequent regeneration. There was some residual tubular atrophy and fibrosis in affected regions. Glomeruli had widespread flattened podocytes with cytoplasmic droplets. The association of these changes with renal uranium concentrations was not provided. Lim et al. (1987) performed a single exposure study in rats, using dosages of 5, 15 or 30 mg/kg administered as intravenous uranyl nitrate. Despite these considerably higher doses, the sequences of clinical and renal pathology findings were consistent with ours. Renal uranium concentration data were not provided, so values cannot be compared with our study. The very high doses of uranium used by Lim et al. (1987) did not appear to result in mortality.

In our study, the degree of proximal tubular necrosis and associated elevations of serum creatinine and BUN concentrations peaked at day seven post-dosing in correlation with peak serum DU concentration. Prerenal azotemia related to dehydration was excluded as a possible explanation for this transient azotemia; this interpretation is supported by the lack of significant mean differences in total protein and globulin values, and hematocrit percentage in the regression model for a dose related effect. Leggett (1983) suggested two mechanisms for explaining this azotemia following exposure to soluble uranium: 1) reduced effective glomular filtration surface area, 2) feedback of solutes across damaged tubules. Ultrastructural examination by electron microscopy (EM) by Lim et al. (1987) and Haley et al. (1982) demonstrated numerous changes in tubular epithelial cells in support of the solute feedback azotemia explanation. Lim et al. (1987) did not provide any EM evidence for the former azotemia explanation (reduced glomular filtration) reporting only that both tubular as well as glomular basement membranes remained intact. However, Haley et al. (1982) did note on EM that glomular visceral epithelium pedicles were broad and flattened in support of direct glomular damage. Intriguingly, in our study the noteworthy dose relationship decline in serum albumin ($p=0.167$) coincided with maximum microscopic evidence of tubular damage, and companying azotemia. This finding taken in consideration with the previously mentioned lack of significant main dose effect on mean group differences for hematocrit, total protein or globulin values suggests that the declining albumin

concentration may be related to glomular loss of albumin and resulting albuminuria due to functional impairment of glomerular podocytes following exposure to soluble uranium. While Diamond et al. (1989) reported proteinuria following low dose exposure of uranyl fluoride in rats, noteworthy changes in serum albumin concentrations have not been previously reported following low dose acute or chronic soluble uranium exposure. Addition evidence of this altered glomerular function is seen in the mesangial proliferation noted by light microscopy in the mid and high dosage groups of this study. This proliferative glomulonephritis (Brun, 1981) is also a unique finding of this study.

In contrast to our study, in which DU was administered IM, Diamond et al. (1989) administered rats eight intraperitoneal doses of uranyl fluoride over a 9-33 day period at two levels, with aggregate doses of 660 or 1329 μg/kg body weight. These led to concentrations of 3.45 or 5.5 μg uranium/g of renal tissue (wet weight) three days after the last exposure (day 36 of the study), which were diminished by ~90% eighteen days later. There was dose-related proximal tubular necrosis and apoptosis (especially affecting the S2 and S3 segments) beginning on day 19 and peaking on day 36, with subsequent regeneration. These investigators noted that tubular injury began at renal concentrations below 1 μg uranium/g.

In addition to the short term studies discussed above, subchronic exposure to uranium has been obtained by dosing in the drinking water. Gilman et al. (1998) administered uranium at levels ranging from 0.96 to 600 mg/L of drinking water to weanling rats for 91 days, and found non-lethal changes in renal tubular epithelium consisting of apical nuclear displacement and cytoplasmic vesiculation along with glomerular capsular sclerosis. Similar changes were seen by McDonald-Taylor et al. (1997) in rabbits dosed at 24 or 600 mg/liter in drinking water for 91 days, with subsequent 45 or 90 day recovery periods. These workers noted sublethal changes to proximal tubular epithelium consisting of loss of brush border, and increased number and size of cytoplasmic vacuoles, lysosomes and mitochondria.

Given the concentration of uranium in the kidney, there has been much interest in determining safe levels. For many years a concentration of 3 μg uranium/gram was considered a chemical toxicity threshold for limiting occupational exposure (Leggett 1989; Russell et al. 1996). While exposure routes, rates and forms of uranium are factors, experimental studies demonstrate severe tubular injury with concentrations of renal uranium well below this threshold (Diamond et al., 1989; Leggett, 1989). Clinical studies suggest a potential for altered proximal tubular function at uranium concentrations well below the 3 μg uranium/gram concentration (Squibb et al., 2005). Our work demonstrates that doses as low as 0.1 mg/kg produce significant renal tubular injury consistent with that of prior studies. Additionally we observed that stress exposure does not worsen or lessen these renal tubular changes and that glomerular damage occurs in conjunction with tubular injury even at the low DU dosages used in this study.

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TABLES

GROUP	STRESS	DU (mg/kg)		SERUM URANIUM (ng U/ml)				TISSUE URANIUM (ug U/g)			
				DAY 1	DAY 3	DAY 7	DAY 30	DAY 1	DAY 3	DAY 7	DAY 30
1	no	0.0	MEAN	0.06	0.06	0.04	0.05	0.01	0.01	0.01	0.01
			SD	0.02	0.07	0.01	0.01	0.00	0.00	0.00	0.01
2	no	0.1	MEAN	0.46				4.99	**2.43**^{D}	**1.11**^{D}	**0.11**^{D}
			SD	0.11				0.71	0.91	0.29	0.01
3	no	0.3	MEAN	1.19	1.29	1.41	**0.15**^{DG}	**16.94**^{G}	**10.77**^{DG}	**3.43**^{DG}	**0.57**^{D}
			SD	0.25	0.64	0.64	0.07	0.96	2.05	1.21	0.17
4	no	1.0	MEAN	**3.60**^{G}	**5.88**^{DG}	**7.71**^{DG}	**0.33**^{DG}	**51.00**^{G}	**22.20**^{DG}	**11.55**^{DG}	**2.15**^{DG}
			SD	1.40	1.49	0.92	0.06	12.80	5.40	0.94	0.84
5	yes	0.0	MEAN	0.04	0.03	0.03	0.04	0.01	0.01	0.01	0.00
			SD	0.01	0.02	0.02	0.02	0.00	0.00	0.00	0.00
6	yes	0.1	MEAN	0.64				4.74	**1.86**^{D}	**1.33**^{D}	**0.11**^{D}
			SD	0.19				1.12	0.25	0.23	0.03
7	yes	0.3	MEAN	**1.43**^{G}	**1.01**^{D}	1.48	**0.13**^{D}	**19.21**^{G}	**15.47**^{DG}	**4.75**^{DG}	**0.72**^{DG}
			SD	0.29	0.14	0.12	0.02	2.93	2.44	0.85	0.28
8	yes	1.0	MEAN	**5.18**^{G}	**7.41**^{G}	**6.35**^{G}	**0.34**^{G}	**53.06**^{G}	**18.81**^{DG}	**12.62**^{DG}	**1.06**^{DG}
			SD	1.56	4.69	2.05	0.07	8.84	0.75	1.23	0.42

TABLE 1 - Study design showing group number, stress exposure, depleted uranium (DU) intramuscular dose, group mean and standard deviation (SD) for serum and kidney uranium concentration on days of collection. N=5 for stressed and unstressed groups on each day. Bolded items statistically different (P-value < 0.05) by one-way ANOVA Dunnett's comparisons with a control (G=comparison across groups for given sample day, group 1 as control; D=comparison across days for given group, day 1 as control).

Analyte	Dose	Day	Stress	Stress + Dose	Stress + Day	Dose + Day	Stress + Dose + Day
BUN	Y	Y	~	~	~	Y	~
Cr	Y	Y	~	~	~	Y	~
P	Y	~	~	~	~	~	~
Ca	~	Y	~	~	~	~	~
TP	~	Y	~	~	~	~	~
Alb	Y	Y	~	~	~	~	~
Glb	~	Y	~	~	~	~	~
ALP	~	Y	~	~	~	Y	~
TBil	~	Y	~	~	~	~	~
K	~	Y	~	~	~	~	~
Cl	~	Y	~	~	~	Y	~
TCO2	Y	Y	~	~	~	Y	~

TABLE 2 - Serum chemistry analytes with significant mean differences for listed effects (Y = $p<0.05$, ~ = $p>0.05$); blood urea nitrogen (BUN), creatinine (Cr), phosphorus (P), calcium (Ca^{++}), total protein (TP), albumin (Alb), globulin (Glb-calculated), alkaline phosphatase (ALP), total bilirubin (TBil), potassium (K^{+}), chloride (Cl^{-}), total carbon dioxide ($TC0_2$).

GROUP	STRESS y/n	DU mg/kg	DAY		BUN mg/dL	Cr mg/dL	P mg/dL	Ca mg/dL	TP d/dL	Alb g/dL	Glb g/dL	ALP uL	TBil mg/dL	K mEq/L	Cl mEq/L	TCO2 mEq/L
1	n	0.0	1	MEAN	22.0	0.4	8.9	11.8	6.9	3.5	3.4	259.0	0.1	6.7	100.4	30.6
				SD	3.3	0.0	0.7	0.3	0.3	0.1	0.2	35.6	0.1	0.8	0.9	1.7
			3	MEAN	23.2	0.4	8.5	11.9	6.9	3.4	3.4	244.6	0.1	6.7	98.8	33.2
				SD	3.4	0.0	0.9	0.2	0.3	0.1	0.3	42.9	0.0	0.5	1.6	1.5
			7	MEAN	22.6	0.4	8.5	11.7	6.7	3.4	3.4	174.8	0.1	6.6	100.2	30.4
				SD	1.5	0.0	0.3	0.2	0.4	0.1	0.3	85.5	0.1	0.6	1.9	1.9
			30	MEAN	25.8	0.4	8.4	11.6	7.0	3.5	3.5	217.6	0.2	7.6	99.6	30.6
				SD	3.6	0.0	1.2	0.5	0.5	0.2	0.5	20.4	0.1	2.0	2.4	2.1
2	n	0.1	1	MEAN	22.2	0.4	8.3	11.7	6.8	3.4	3.3	201.4	0.1	6.1	100.6	30.2
				SD	0.8	0.0	0.7	0.3	0.2	0.2	0.1	23.4	0.0	0.5	2.3	3.6
			3	MEAN	* 35.7 GD	* 0.7 D	7.7	11.4	6.7	3.4	3.3	207.0	0.1	7.0	102.3	29.7
				SD	6.2	0.1	0.6	0.4	0.4	0.1	0.3	34.0	0.0	1.1	2.2	2.9
			7	MEAN	25.6	0.4	8.5	11.2	6.6	3.3	3.3	210.2	0.1	6.9	100.4	31.8
				SD	4.7	0.0	1.2	0.4	0.5	0.2	0.3	50.8	0.1	0.7	1.3	0.8
			30	MEAN	26.2	0.4	7.8	11.6	7.1	3.5	3.5	227.2	0.1	7.0	99.2	30.0
				SD	5.0	0.0	1.4	0.4	0.4	0.2	0.3	55.3	0.1	1.2	1.8	3.2
3	n	0.3	1	MEAN	24.4	0.4	7.7	11.7	7.0	3.5	3.5	196.2	0.1	6.1	99.6	31.2
				SD	3.4	0.1	0.6	0.3	0.3	0.1	0.2	43.2	0.0	0.4	3.2	1.8
			3	MEAN	* 46.0 G	* 1.0 G	7.4	11.8	6.8	3.4	3.4	232.6	0.1	6.9	102.4	29.4
				SD	3.5	0.2	0.6	0.7	0.3	0.1	0.2	25.4	0.0	0.7	1.5	2.6
			7	MEAN	55.2 D	1.0	8.1	11.3	6.6	* 3.2 D	3.3	208.4	0.1	6.6	98.2	32.0
				SD	36.9	0.8	0.8	0.3	0.3	0.2	0.2	20.6	0.1	0.2	1.6	2.0
			30	MEAN	23.2	0.4	6.7	11.5	7.2	3.6	3.5	248.2	0.2	5.9	99.6	32.8
				SD	1.1	0.0	0.6	0.4	0.5	0.2	0.4	46.3	0.1	0.4	1.1	2.4
4	n	1.0	1	MEAN	24.2	* 0.5 G	8.7	11.6	6.9	3.4	3.5	220.2	0.1	7.0	101.0	28.8
				SD	4.9	0.1	0.9	0.5	0.4	0.2	0.3	37.4	0.0	1.6	2.3	1.9
			3	MEAN	* 56.0 GD	* 1.7 GD	7.1	11.9	6.6	* 3.2 G	3.4	241.2	0.1	7.0	104.6	27.6
				SD	6.0	0.3	0.6	0.4	0.5	0.0	0.5	42.5	0.0	0.4	1.3	3.3
			7	MEAN	* 104.8 GD	* 2.2 GD	7.2	11.1	6.5	3.2	3.2	227.2	0.1	6.2	98.8	31.2
				SD	29.1	0.5	0.7	0.3	0.4	0.1	0.3	27.5	0.1	0.6	2.6	1.3
			30	MEAN	27.0	0.4	7.7	11.7	7.1	3.5	3.6	250.2	0.1	6.3	100.0	30.4
				SD	2.5	0.0	0.9	0.5	0.5	0.2	0.4	16.6	0.0	0.7	1.2	2.7
5	y	0.0	1	MEAN	22.0	0.4	7.7	11.9	6.9	3.4	3.5	271.0	0.1	5.7	99.5	30.8
				SD	2.6	0.1	0.6	0.4	0.2	0.2	0.2	58.2	0.0	0.6	1.0	2.1
			3	MEAN	23.8	0.4	8.0	11.7	6.9	3.5	3.4	245.0	0.1	6.5	99.2	33.6
				SD	1.1	0.0	0.6	0.2	0.2	0.0	0.2	57.0	0.0	0.5	2.2	4.4
			7	MEAN	23.4	0.4	8.3	11.9	6.9	3.5	3.4	213.8	0.1	6.6	99.0	31.2
				SD	1.8	0.0	0.6	0.6	0.3	0.1	0.2	42.0	0.1	0.6	1.2	2.0
			30	MEAN	22.6	0.4	8.0	11.7	7.2	3.5	3.6	213.2	0.2	6.7	99.8	31.6
				SD	1.1	0.0	1.3	0.5	0.2	0.1	0.2	30.5	0.1	1.0	0.4	1.7
6	y	0.1	1	MEAN	23.3	0.4	8.3	11.6	6.9	3.6	3.3	205.3	0.1	6.8	102.0	30.3
				SD	2.1	0.0	0.9	0.4	0.3	0.2	0.2	28.6	0.0	1.2	0.8	2.8
			3	MEAN	* 36.8 GD	* 0.7 D	7.8	11.7	6.8	3.4	3.4	239.0	0.1	6.9	102.6	29.8
				SD	4.0	0.1	0.4	0.5	0.2	0.1	0.2	43.0	0.0	1.1	1.1	2.9
			7	MEAN	27.2	0.5	8.3	11.3	6.6	3.3	3.3	213.0	0.2	7.1	99.8	31.0
				SD	1.6	0.1	1.2	0.3	0.1	0.1	0.2	28.0	0.2	0.7	1.1	1.6
			30	MEAN	23.6	0.4	7.2	11.5	7.1	3.6	3.6	203.0	0.2	6.5	99.4	31.0
				SD	1.3	0.0	0.5	0.2	0.3	0.1	0.3	26.0	0.0	0.5	0.9	3.2
7	y	0.3	1	MEAN	23.7	0.4	8.4	11.4	6.7	3.5	3.3	261.5	0.1	6.9	101.3	28.2
				SD	3.4	0.0	1.1	0.6	0.3	0.2	0.2	33.1	0.0	1.9	1.6	2.1
			3	MEAN	* 50.2 GD	* 1.1 GD	7.5	11.9	6.9	3.4	3.5	244.3	0.2	6.6	102.2	29.5
				SD	5.0	0.1	0.9	0.5	0.3	0.1	0.2	41.8	0.2	0.3	1.3	1.2
			7	MEAN	* 62.2 D	* 1.1 D	8.2	11.4	6.6	3.2	3.4	231.0	0.2	7.2	97.4	33.2
				SD	21.9	0.4	1.4	0.4	0.4	0.1	0.4	31.9	0.1	1.0	1.5	1.1
			30	MEAN	26.6	0.4	7.6	11.6	7.1	3.5	3.6	241.6	0.1	6.9	99.4	30.8
				SD	3.6	0.0	1.2	0.5	0.5	0.2	0.4	44.6	0.1	1.3	1.3	2.9
8	y	1.0	1	MEAN	25.0	* 0.5 G	8.2	11.6	6.8	3.4	3.5	247.6	0.1	6.9	100.0	28.6
				SD	1.4	0.1	1.4	0.5	0.1	0.1	0.1	32.1	0.0	2.5	2.3	3.2
			3	MEAN	* 61.0 G	* 1.8 GD	7.9	12.1	6.5	* 3.2 G	3.3	230.8	0.1	6.7	104.2	27.4
				SD	11.6	0.1	1.0	0.2	0.3	0.2	0.3	22.2	0.0	1.2	1.9	1.5
			7	MEAN	* 110.0 GD	* 2.4 GD	7.3	11.5	6.7	3.3	3.5	222.2	0.2	6.2	98.6	31.4
				SD	44.5	1.1	1.7	0.6	0.3	0.2	0.2	39.2	0.0	0.8	2.5	3.0
			30	MEAN	26.8	* 0.5 G	7.8	11.5	6.9	3.5	3.4	252.0	0.1	6.9	100.6	32.2
				SD	2.4	0.1	0.8	0.6	0.4	0.1	0.3	30.8	0.1	1.2	1.7	2.2

TABLE 3 - Group mean and standard deviation (SD) results sorted by day for serum chemistry analytes with significant effect related difference as shown in Table 2; blood urea nitrogen (BUN), creatinine (Cr), phosphorus (P), calcium (Ca^{++}), total protein (TP), albumin (Alb), globulin (Glb-calculated), alkaline phosphatase (ALP), total bilirubin (TBil), potassium (K^{+}), chloride (Cl^{-}), total carbon dioxide ($TC0_2$); starred results in BUN, Cr, Alb column statistically different (P-value < 0.05) by one-way ANOVA Dunnett's comparisons with a control (G=comparison across groups for given sample day, group 1 as control; D=comparison across days for given group, day 1 as control).

FIGURES

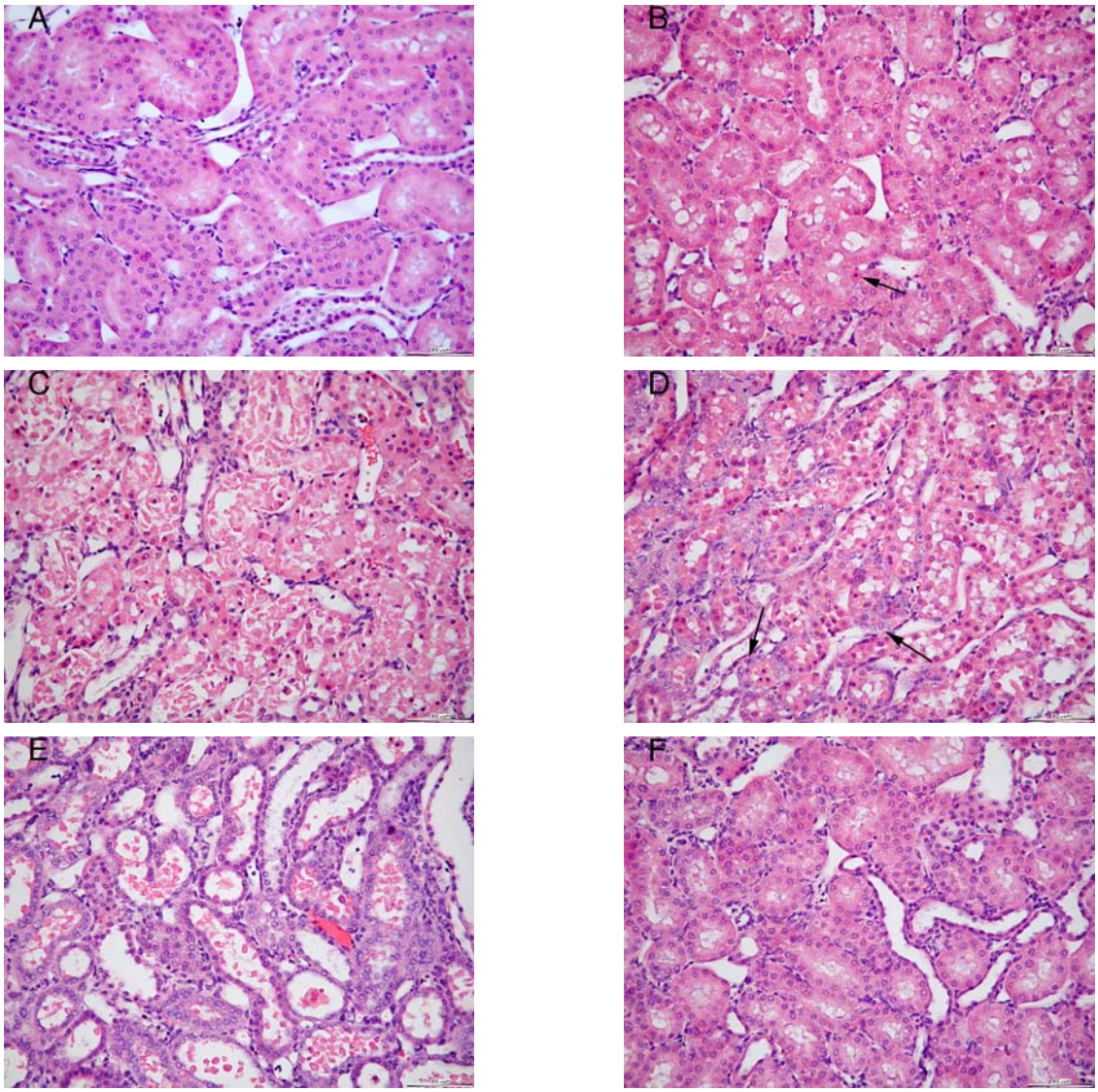

FIGURE 1 - Outer stripe of renal medulla, H&E stain. (**A**) Stressed control rat, intact tubular epithelium, lesion grade 0. (**B**) Early injury to proximal tubular epithelium, with swelling and cytoplasmic vacuolization, occasional recent necrosis (arrow) is seen. Day 1, stress, 1 mg/kg DU, lesion grade 1. (**C**) Extensive necrosis and sloughing of proximal tubular epithelium. Day 3, stress, 1 mg/kg DU, lesion grade 2. (**D**) Extensive necrosis of proximal tubular epithelium with focal regions of faintly basophilic early regenerative cells (arrows). Day 3, stress, 0.1 mg/kg DU, lesion grade 3. (**E**) Flattened, basophilic regenerating epithelium lines most of the proximal tubules. Day 7, no stress, 0.3 mg/kg DU, lesion grade 4. (**F**) Advanced regeneration of proximal tubular epithelium. Day 30, no stress, 0.3 mg/kg DU, lesion grade 5.

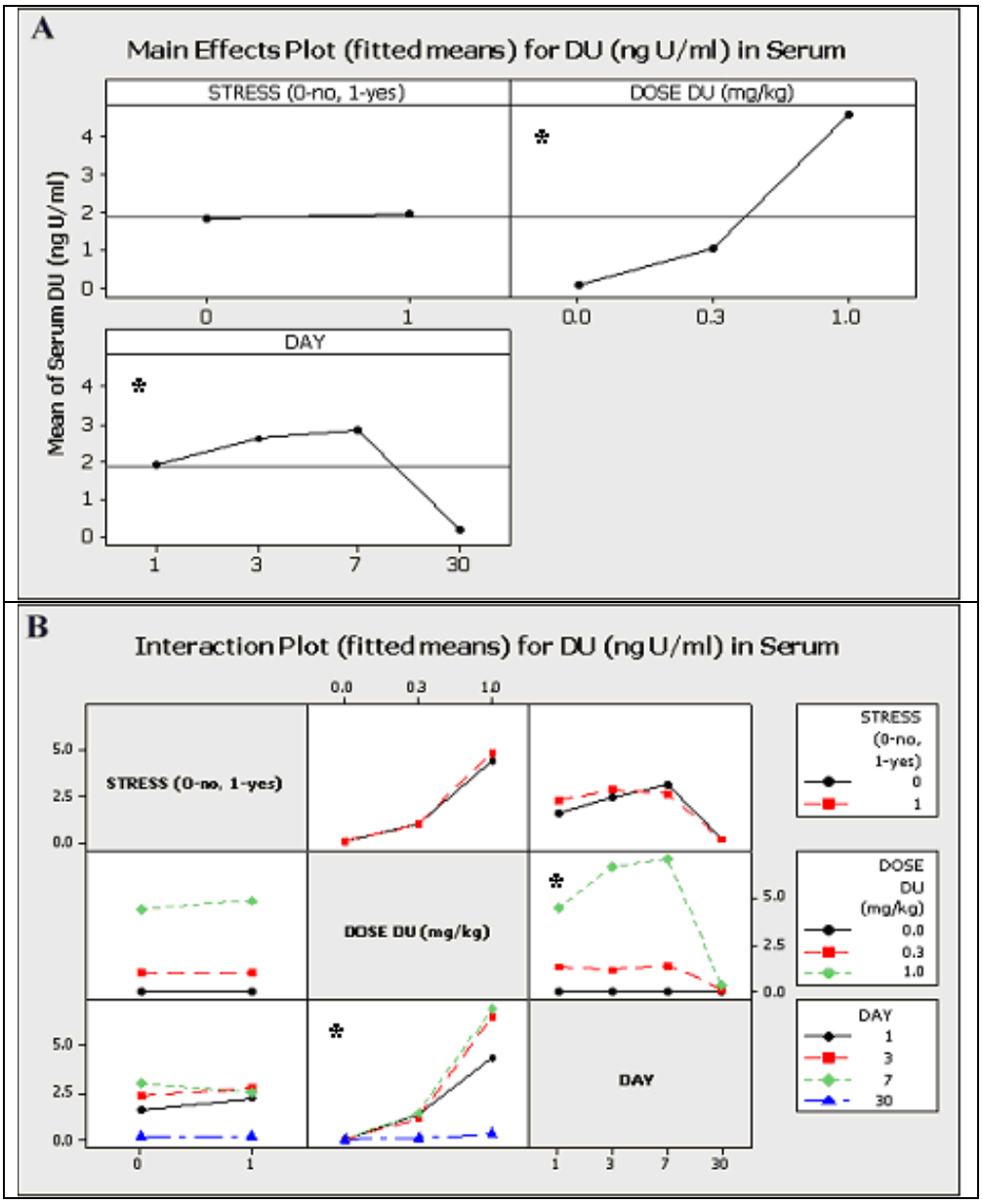

FIGURE 2 – Stress, dose, and day effects on mean serum uranium concentration. (A) Main effects (* dose and day significantly different, $p<0.05$); (B) Interactive effects (* dose-day significantly different, $p<0.05$).

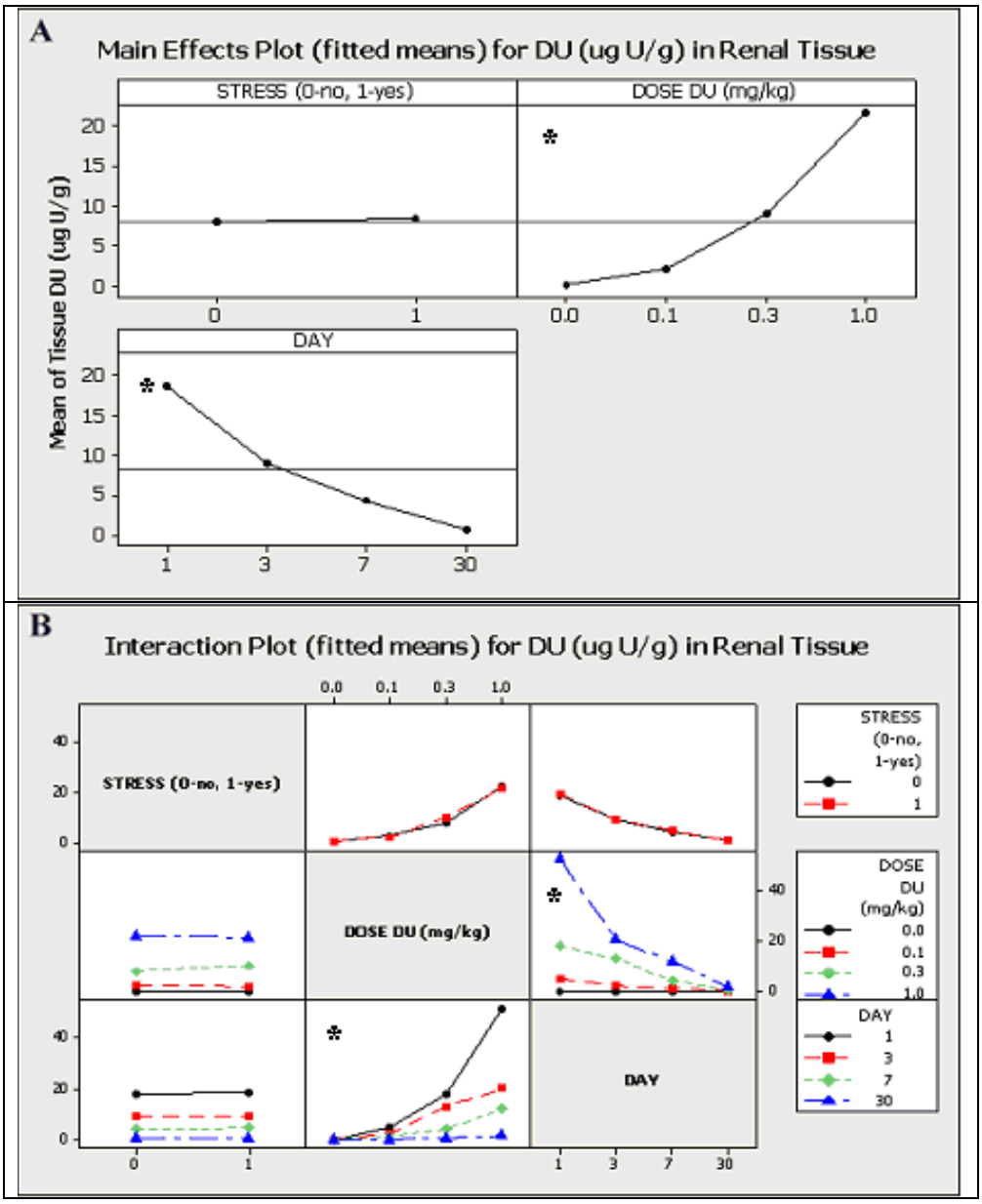

FIGURE 3 - Stress, dose, and day effects on mean tissue uranium concentration. (A) Main effects (* dose and day significantly different, $p<0.05$); (B) Interactive effects (* dose-day significantly different, $p<0.05$).

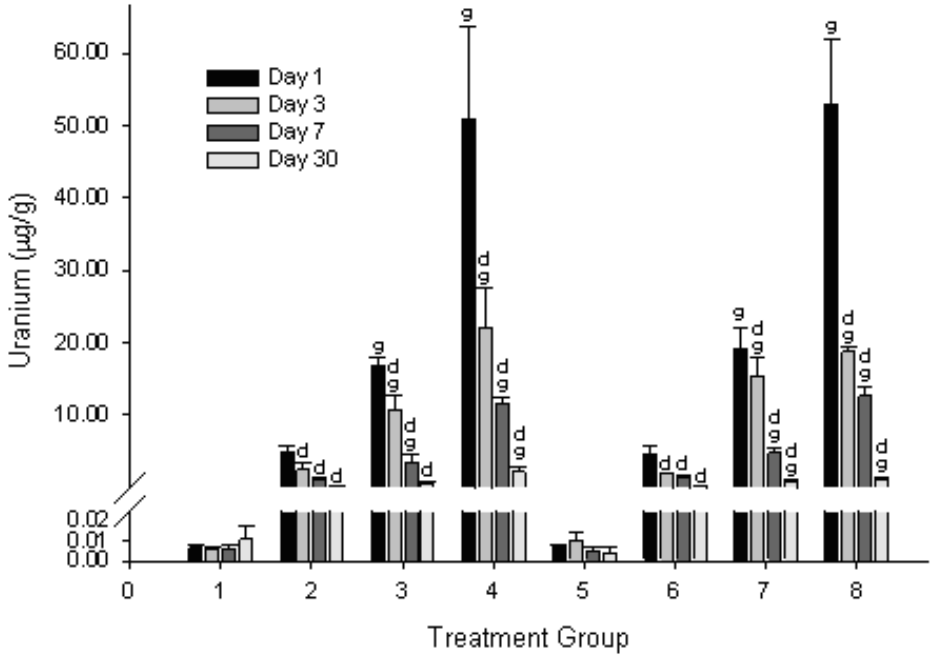

FIGURE 4 - Mean uranium concentration in renal tissue showing dose dependent relationship and similarity of response between no-stress (groups 1-4) and stress (groups 4-8); statistically different columns shown with superscripts (g=comparison across groups for given sample day, group 1 as control; d=comparison across days for given group, day 1 as control [P-value < 0.05, one-way ANOVA Dunnett's comparisons with control]).

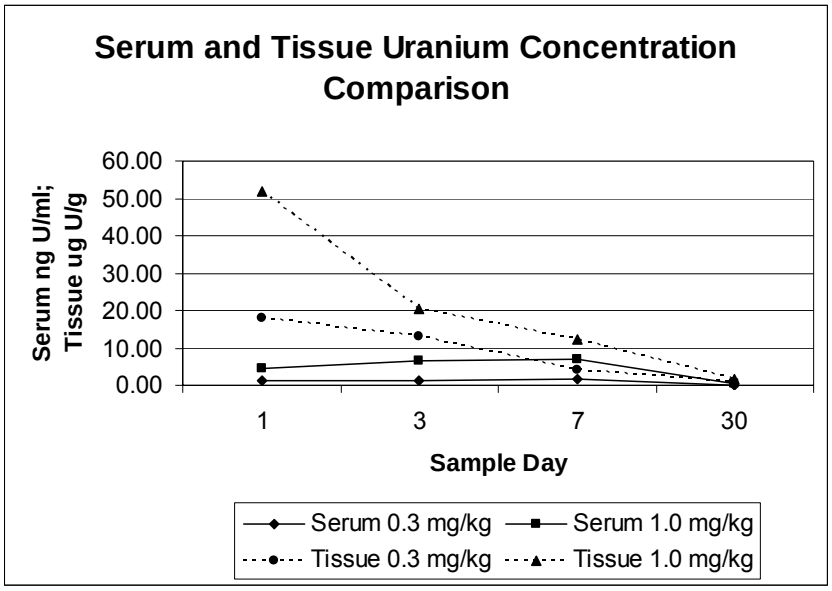

FIGURE 5 - Comparison between mean serum (ng/ml) and tissue (ug/g) uranium concentration over study period for the dosage groups receiving 0.3 mg/k DU (mean of groups 3 and 7) and 1.0 mg/kg DU (mean of groups 4 and 8).

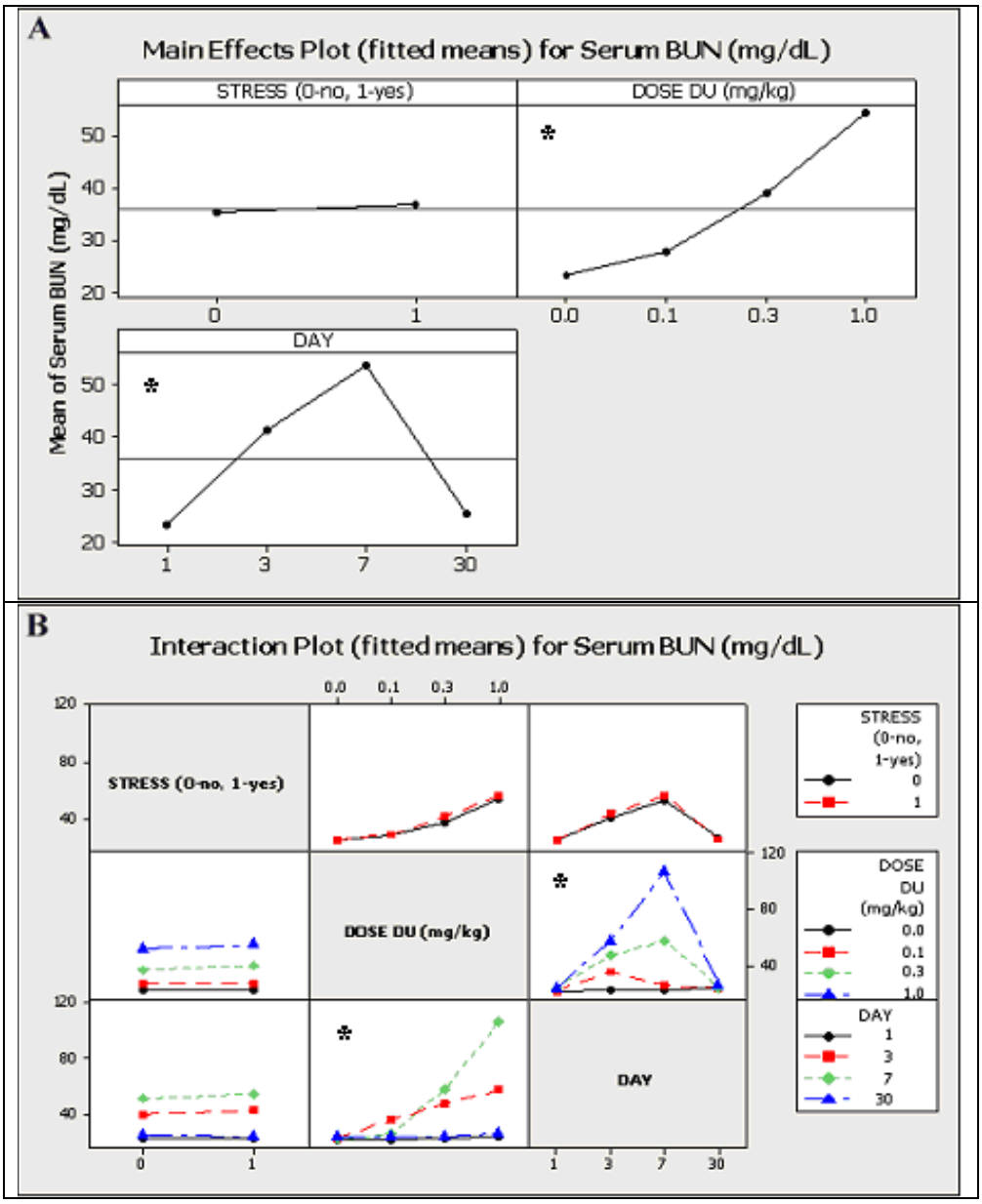

FIGURE 6 – Stress, dose, and day effects on mean serum blood urea nitrogen concentration. Main effects (* dose and day significantly different, $p<0.05$); (B) Interactive effects (* dose-day significantly different, $p<0.05$).

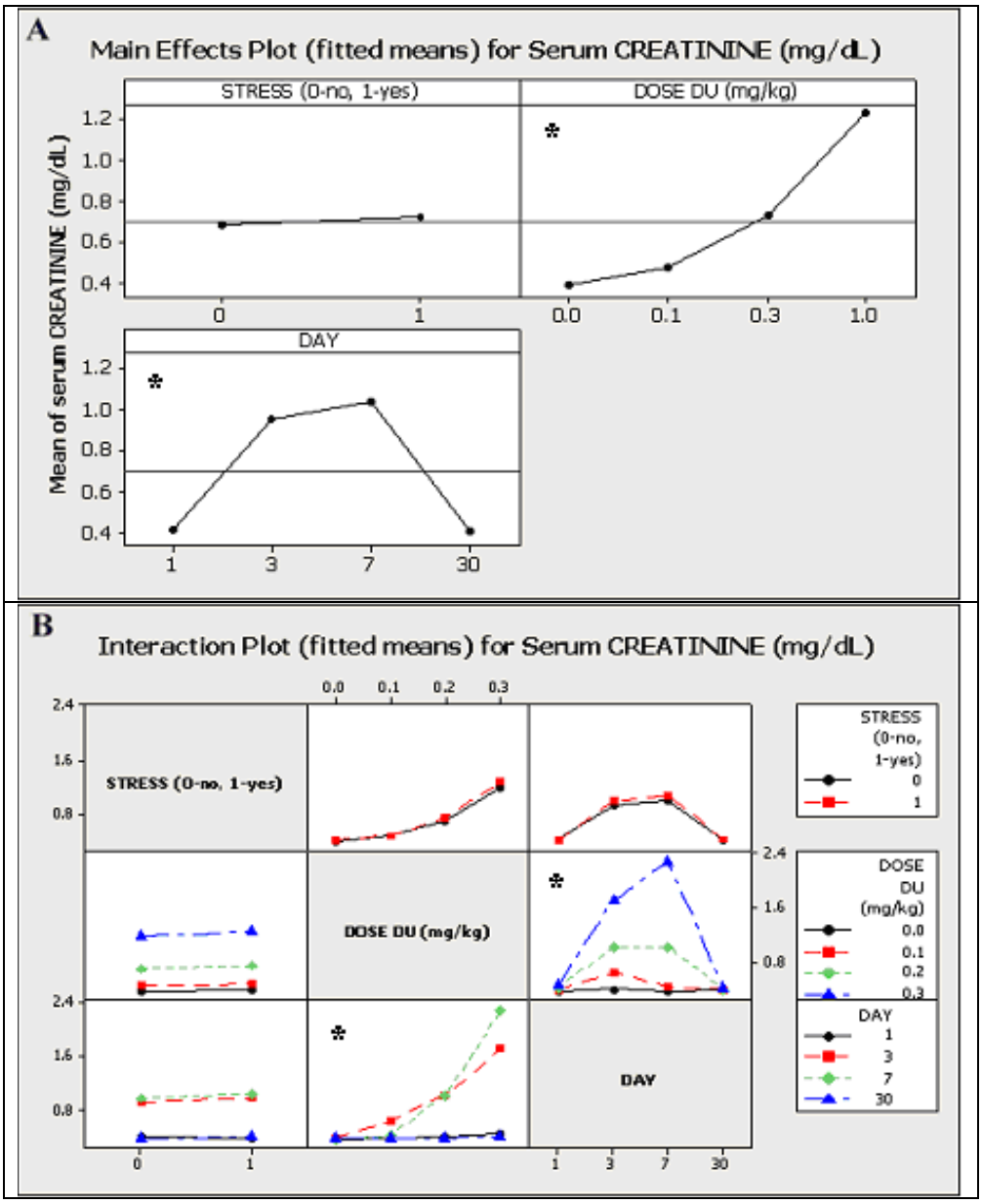

FIGURE 7 – Stress, dose, and day effects on mean serum creatinine concentration. (A) Main effects (* dose and day significantly different, $p<0.05$); (B) Interactive effects (* dose-day significantly different, $p<0.05$).

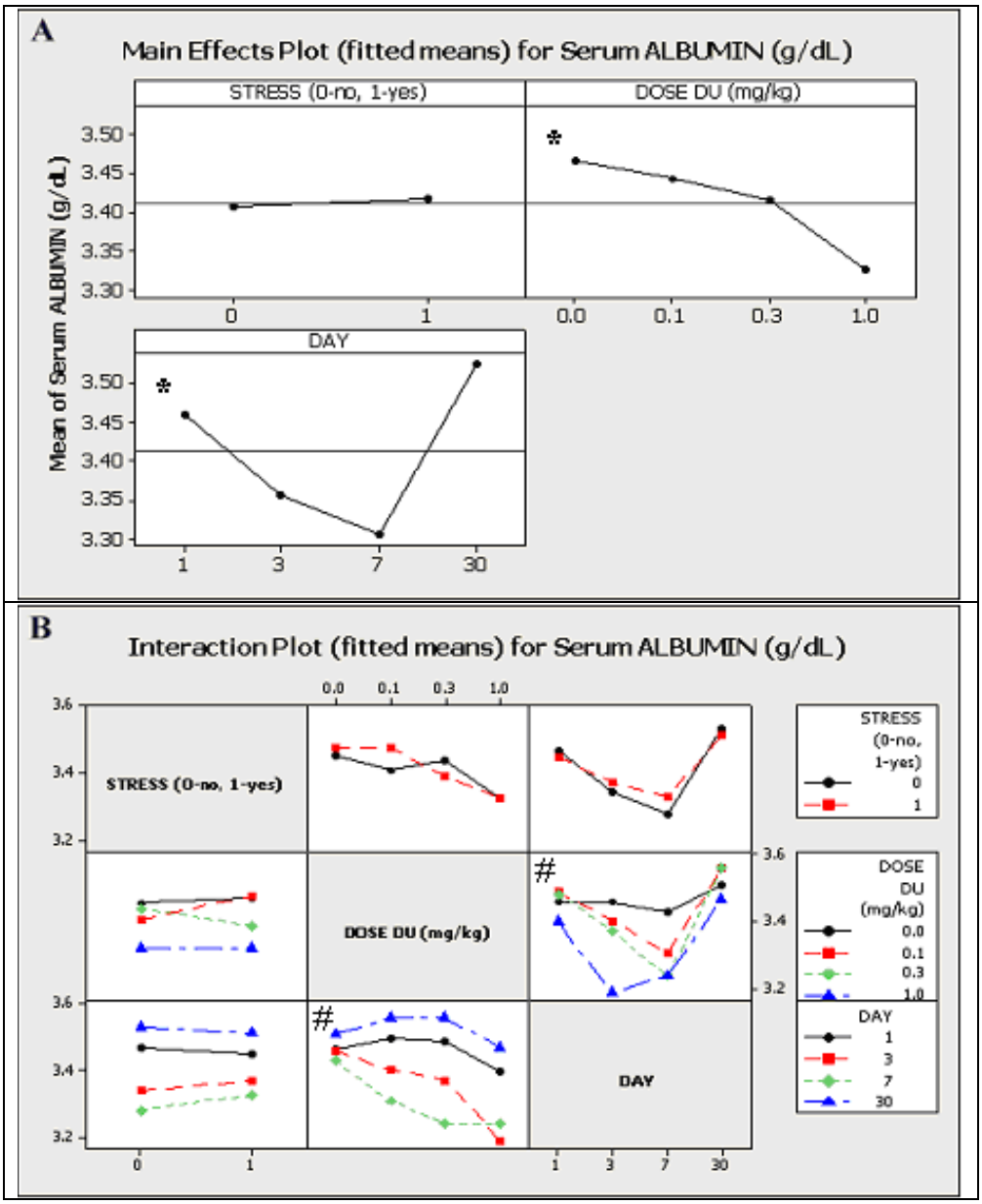

FIGURE 8 - Stress, dose, and day effects on mean serum albumin concentration. (A) Main effects (* dose and day significantly different, $p<0.05$); (B) Interactive effects (# dose-day approaching significance, $p=0.167$).

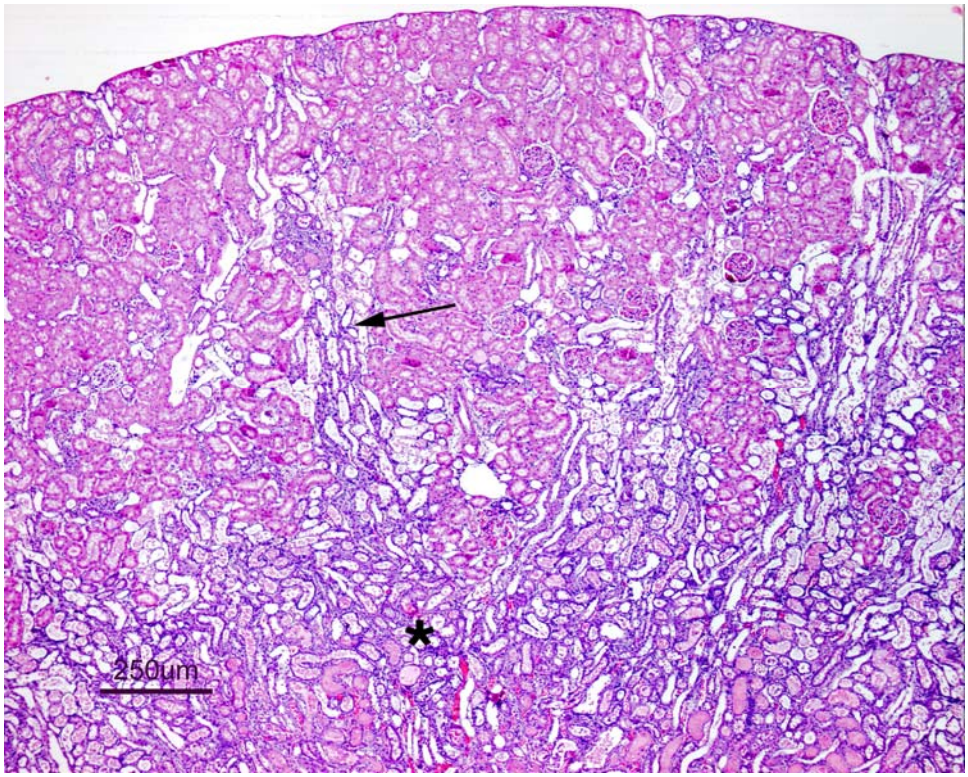

FIGURE 9 - Blue-staining regenerating tubular epithelial cells indicate regions affected in uranium nephrotoxicity. This includes a band involving deep cortex and outer stripe of the medulla (*) and medullary rays in cortex (arrow). Day 7, 1.0 mg/kg DU, H&E stain.

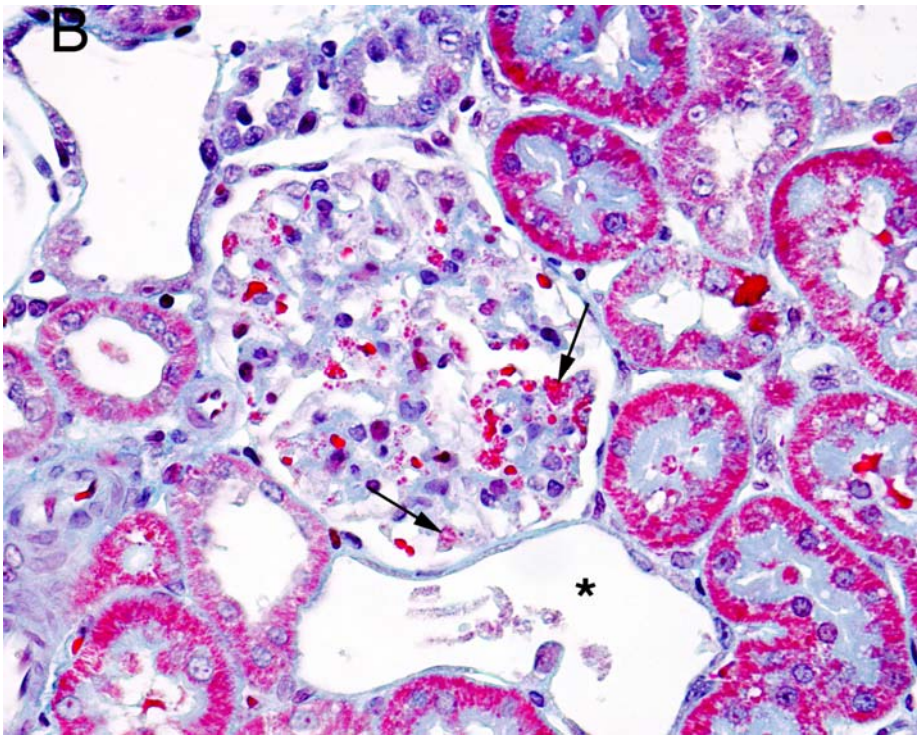

FIGURE 10 - Compared to a control (A), glomerulus from a rat treated with 1.0 mg/kg DU shows expanded mesangium containing red-staining proteinaceous droplets (arrows). An adjacent proximal tubule is denuded of epithelium (*). Day 7, Masson's trichrome stain.

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The effect of stress on the acute neurotoxicity of the organophosphate insecticide chlorpyrifos

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Abstract

A study was conducted to determine if multiple exposures to several stress paradigms might affect the anticholinesterase effect of subsequently administered organophosphate insecticide chlorpyrifos. Male Sprague–Dawley rats were subject to daily periods of restraint, swimming, a combination of the two, or neither of the two (controls) ($n=8$/group) for 5 days per week over a six-week period. The most profound stress, as measured by reduced body weight gain and elevated levels of plasma corticosterone, was swimming. On day 39 of the study, shortly after the daily stress episode, one half of the rats in each group ~~were~~ dosed with 60 mg/kg chlorpyrifos subcutaneously. This had no effect on subsequent levels of plasma corticosterone. There were no stress-related differences in the degree of chlorpyrifos-induced inhibition of brain acetylcholinesterase in animals sacrificed on day 43.

Keywords: Organophosphates; Chlorpyrifos; Stress; Acetylcholinesterase inhibition; Brain; Rat

Introduction

Imbalance between environmental demands for survival and the individual's capacity to adapt to these is defined as stress (Marshall et al., 2000; Lazarus and Folkman, 1984; Sapolsky, 1992), and such responses are needed in adapting to demands of ever-changing circumstances. Individuals react to stress by shifting resources from other biological activities (such as reproduction or growth) toward survival. The degree of these responses relates to the intensity and duration of the stress. Milder forms of stress draw on reserve resources, but severe acute or chronic stress may impact on critical metabolic pathways and thus negatively alter homeostatic metabolic events. This results in decreased capability to adapt to changes in ambient temperatures, resist infectious agents, or tolerate exposure to natural and synthetic toxicants.

Adrenocortical trophic hormone-mediated glucocorticoid secretions from the adrenal cortex, such as corticosterone, induced by a complex of brain and hypothalamic–pituitary–adrenal axis reactions, along with catecholamines, are major mediators of stress effects (Sapolsky, 1992, 1996, 2000). Physiological effects of such stress-induced hormonal changes include diversion of energy to the exercising muscles (such as by mobilization of stored energy and gluconeogenesis), enhanced cardiovascular tone increasing substrate delivery to muscle and brain, acute stimulation of immune function, and sharpened cognition with increased cerebral glucose utilization.

While beneficial for a short period of time, chronically elevated blood levels of glucocorticoids, as seen with prolonged or severe stress, provoke enhanced demands on the body's resources. This is manifest by protein catabolism, hyperglycemia, immune suppression, and altered immunoregulation with enhanced susceptibility to infection, depression, altered mental performance, and decreased hippocampal volume (Agarwal and Marshall, 1998; McEwen and Stellar, 1993). Under such conditions, stress is thought to enhance development of disease, including that of the nervous system (Sapolsky, 1992, 1996). Experimental studies in rodents indicate that prolonged or severe stress may damage the brain. Most prominently, stress-induced high blood levels of glucocorticoids mediate deleterious effects in the hippocampus, a region rich in receptors for

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these hormones (McEwen, 2001; Sapolsky, 1992, 2000). The elevated glucocorticoids act via several pathways, prominently interacting with excitatory amino acid neurotransmitters (glutamate) in the evolution of the neuropathological effects (Magarinos and McEwen, 2000; Sapolsky, 1996, 2000).

In addition to possible direct effects on brain, stress hormones may enhance effects of other neuropathic agents. As regards neurotoxicity, there have been a number of investigations of a possible role of stress in enhancing central effects of ~~antic-holinesterase~~ chemicals, arising from suggestions that this combination may have played an etiologic role in components of the Gulf War Illnesses (Abdel-Rahman et al., 2002; Institute of Medicine, 2003). Most of these studies have focused on stress-associated reduction of the blood–brain barrier efficiency, thus allowing some commonly used drugs, in particular the carbamate pyridostigmine bromide, to elicit central cholinergic effects in laboratory animals. Investigations of whether stress affects the antiesterase effects of organophosphates are few. Single or multiple daily stress exposures had no effect on the acute toxicity of paraoxon or chlorpyrifos (Pung et al., 2006; Shaikh and Pope, 2003), and a model of chronic stress had no effect on delayed neurotoxicity induced by tri-*ortho*-tolyl phosphate (Jortner et al., 2005). We report the results of a study of the effect of multiple stressors administered over a six-week period to rats on the acute neurotoxicity of the organophosphate insecticide chlorpyrifos. The latter is a commonly employed lipophilic insecticide, which can penetrate the blood–brain barrier.

Methods

Animals. Male Sprague–Dawley (Harlan Sprague–Dawley, Dublin, VA) rats were used in this study. They were 66–74 days of age at the onset and were single-cage housed in a standard laboratory animal room at 22–24 °C with a 12-h light/dark cycle. The animals had free access to Harlan Teklad 2018 Rodent Diet and tap water. Animal experiments adhered to the principles stated in the guide for the care and use of laboratory animals (National Research Council, 1996) and were reviewed and approved by the Virginia Tech Animal Care and Use Committee.

Stress. The rats were divided into four groups ($n=8$/group), each exposed to a different stress paradigm over the six-week duration of the study (Fig. 1), as follows.

SUN	MON	TUES	WED	THURS	FRI	SAT
		Day-6	Day -5	Day -4	Day -3	Day -2
		Acclimation to blood collection procedures			BW blood (CORT)	
Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
	stress	stress	stress	stress	stress BW blood (CORT)	
Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
	stress	stress	stress	stress	stress BW blood (CORT)	
Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19
	stress	stress	stress	stress	stress BW blood (CORT)	
Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26
	stress	stress	stress	stress	stress BW	
Day 27	Day 28	Day 29	Day 30	Day 31	Day 32	Day 33
	stress	stress	stress	stress	stress BW blood (CORT and AChE)	
Day 34	Day 35	Day 36	Day 37	Day 38	Day 39	Day 40
	stress	stress	stress	stress motor activity	stress BW CPF dosing	
Day 41	Day 42	Day 43				
	stress BW motor activity	sacrifice blood (CORT) brain (AChE)				

Abbreviations-
BW = body weight
blood = blood collection
AChE =acetylcholinesterase
CORT = corticosterone
CPF = chlorpyrifos

Fig. 1. Schedule of the study showing days for stress administration, sample collection and sacrifice.

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Group 1 (control) — These rats were subject to daily routine handling for 5 days per week and comprised the stress control group.

Group 2 (restraint only) — These rats were restrained in acrylic tubes 6 cm in diameter and 22 cm in length for 30 min per day, 5 days per week.

Group 3 (restraint+swimming) — Rats were restrained as noted above on days 1 through 4 of each week and were swum as noted below on the fifth day.

Group 4 (swimming only) — Rats swam for 30 min/day in tap water at 22–24 °C for 5 days per week. Following each swimming episode, the rats were gently towel dried and placed under a warming lamp.

Prior to the beginning of the stress exposure and first blood collection, the rats were acclimated to handling for the bleeding procedure for three consecutive days. Day 0 was the first day of stress exposure, and the rats were stressed Monday through Friday for 6 weeks. Stress effects were measured by weekly determination of body weight changes and by periodic assay of corticosterone levels in blood plasma, as noted in Fig. 1. For the latter, blood was drawn from the orbital sinus, under isoflurane general anesthesia within 10 min of the cessation of the stress. Corticosterone levels were determined by radioimmunoassay, using a commercially available kit (Corticosterone 125[I] Radioimmunoassay Kit, ICN Diagnostics, Costa Mesa, CA).

Organophosphate effects

Since a focus of this study was to determine stress effects on ~~organophosphate~~ acute neurotoxicity, chlorpyrifos was administered to 4/8 rats of each group in a single subcutaneous dose of 60 mg/kg, immediately after the stress exposure on day 39 (Fig. 1). Controls received an equivalent volume of the corn oil vehicle. Thus, each of the four stress groups was further divided into chlorpyrifos-exposed and non-exposed cohorts ($n=4$). This exposure to chlorpyrifos took place after the bulk of the stress exposures, and the rats were sacrificed on day 43 (Fig. 1). Effects of stress on acetylcholinesterase were determined in whole blood samples obtained by orbital bleeding on day 32. Effects of stress and chlorpyrifos on acetylcholinesterase were assessed by measuring whole brain enzyme activity, normalized to mg protein, in samples obtained at sacrifice. The latter was performed by CO_2 inhalation. ~~Acetylcholinesterase~~ activities were determined using the microplate method of Correll and Ehrich (1991).

Clinical evaluation

In addition to the weekly body weight determinations noted above, motor activity was assessed using the San Diego Instrument Co. cage rack automated motor activity unit. This measures fine (non-ambulatory) and horizontal (ambulatory) movements, and total motor activity. The rats were evaluated for this behavior for 30 min during the dark (most active) cycle on day 38 (prior to chlorpyrifos dosing) and on day 42 (3 days after dosing) (Fig. 1).

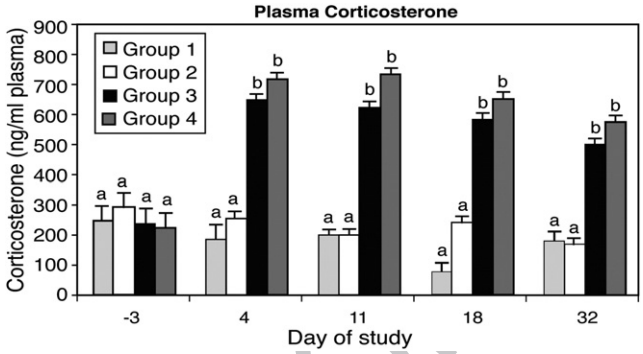

Fig. 3. Elevation of plasma corticosterone relative to controls (Group 1) was seen at all intervals measured in restraint+swimming (Group 3) and swimming only (Group 4) rats ($p<0.05$, indicated by b). The restraint only (Group 2) rats were not different from the controls (a) on these days. Day −3 was prior to initiation of stress. The samples were drawn on days in which the restraint+swimming (Group 3) animals had their weekly swim ($n=8$/group).

Statistical methods

Data from measured responses were subjected to analysis of variance with separation by Bonferroni-corrected multiple comparisons using the SAS System (version 8.02, SAS Institute, Cary, NC). Significance was considered when $p<0.05$. Data are presented in graphic or tabular form as mean±standard error of the mean.

Results

Stress effects

Stress was measured by changes in body weight gain and plasma corticosterone over the course of the study, as shown in Fig. 2. There was progressive body weight increase in all stress groups ($n=8$/group). This was greatest for controls (Group 1) and less for the three groups exposed to stress. The difference was statistically significant only for the swim rats (Group 4) over the 18- to 42-day period (Fig. 2). Chlorpyrifos had no effect on body weight gain measured on day 42 (Fig. 2).

Similarly, plasma corticosterone levels were significantly elevated in the restraint-swim (Group 3) and swim (Group 4) animals. This was noted in blood samples obtained shortly after

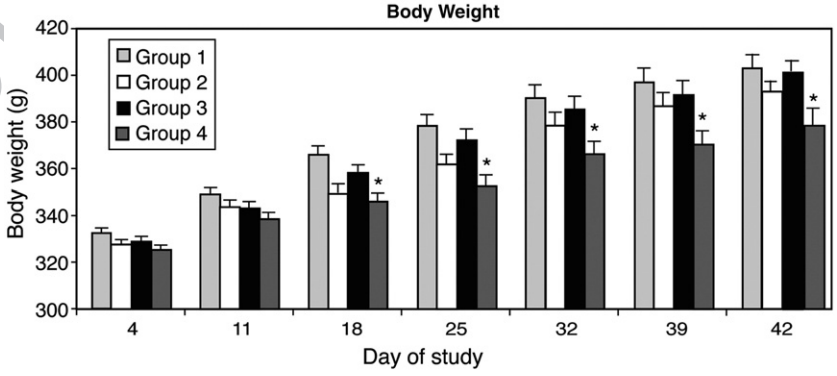

Fig. 2. Changes in body weight demonstrate that all four experimental groups gained weight over the course of the study. This was greatest for Group 1 (controls). The rate of growth was significantly less (* indicates $p<0.05$) than controls in Group 4 (swimming only) on days 18, 25, 32, 39 and 42 ($n=8$/group). Day 42 includes rats dosed and not dosed with chlorpyrifos. The latter had had no effect on this parameter.

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t1.1 Table 1
Mean (±SEM, $n=8$) motor activity measurements by stress treatment group
t1.2 before (day 38) dosing with chlorpyrifos

t1.3 Group[a]	Ambulatory	Non-ambulatory	Total
t1.4 1	131.43±13.37	228.43±34.31	359.86±44.93
t1.5 2	107.25±12.50	272.50±32.09	379.75±42.03
t1.6 3	136.00±13.37	267.00±34.31	403.00±44.93
t1.7 4	127.25±12.50	292.38±32.09	419.63±42.03

t1.8 [a]$n=8$/group, values are numbers of beam breaks in the 30-min test period

the stress episodes on days 4, 11, 18 and 32 (Fig. 3). These data were obtained prior to chlorpyrifos exposure. It should be noted that the values for Group 3 were obtained after the single swim stress for each week (Fig. 1). Those values are similar to ones obtained from the swim only rats (Group 4, Fig. 3). Stress had no effect on motor activity as measured on day 38 (Table 1) and on whole blood acetylcholinesterase activity on day 32 (data not shown).

Stress–chlorpyrifos interactions

Administration of chlorpyrifos to 4/8 rats per group on day 39 had no effect on plasma corticosterone levels on day 43, whether or not intervening stress was administered (Fig. 4). Similar swim-induced elevations were seen in both chlorpyrifos and non-chlorpyrifos-exposed rats. Since samples for corticosterone were taken the day after the swim stress, elevation of this hormone noted in the swimming rats (Group 4) was not noted in the restraint-swim rats (Group 3). In addition, none of the stress paradigms affected the ability of chlorpyrifos to diminish activity of brain acetylcholinesterase, as measured in tissue obtained on the day 43 sacrifice (Fig. 5). No clinical evidence of cholinergic effects was noted in any rats at any time after dosing with 60 mg/kg chlorpyrifos. There was a trend towards a reduction in motor activity ($p=0.0737$ for main effect of chlorpyrifos, Table 2) in all chlorpyrifos-exposed subgroups.

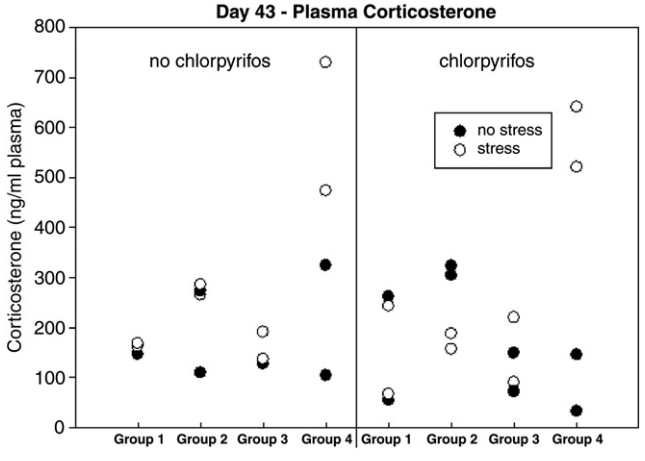

Fig. 4. Chlorpyrifos administered on day 39 had no effect on plasma corticosterone levels on day 43. Groups (1—control, 2—restraint only, 3—restraint+swimming, 4—swimming only) are further divided into rats receiving and not receiving stress on day 43, with corticosterone levels provided for each animal. The no stress rats of Group 4 were similar to controls, although these animals had been swum the previous day (Fig. 1).

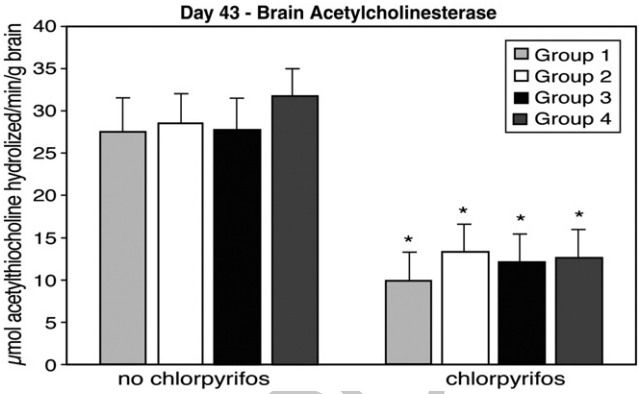

Fig. 5. Chlorpyrifos administered on day 39 resulted in significant inhibition of brain acetylcholinesterase on day 43 (*$p<0.05$). This is not affected by any stress paradigm ~~any stress paradigm~~ (Group 1—routine handling, Group 2—restraint, Group 3—restraint+swimming, Group 4—swimming only).

Discussion

There has been considerable interest in the role of stress in exacerbating neurotoxic effects, in particular as it relates to cholinesterase inhibition. Repeated acute stress has been reported to diminish acetylcholinesterase activity and transcription of genes coding for acetylcholinesterase (Kaufer et al., 1998). The role of stress-related alteration of the action of anticholinesterase agents has been extensively studied for the carbamate pyridostigmine bromide, stemming from reviews of Gulf War (1990–1991) illnesses. The latter have suggested interaction between operational stress and exposure to this drug may have led to unforeseen health consequences (Hanin, 1996; Institute of Medicine, 2003). Pyridostigmine bromide was used as pre-exposure prophylaxis against nerve agents during the Gulf War. The drug is a transient inhibitor of peripheral acetylcholinesterase, and its charged quaternary nitrogen restricts movement across the blood–brain barrier. A study by Friedman et al. (1996) indicated that 10 min of forced swimming in mice allowed pyridostigmine to penetrate the blood–brain barrier and inhibit brain acetylcholinesterase. However, a number of subsequent studies were unable to replicate these findings, using a variety of stressors such as restraint, forced

t2.1 Table 2
Mean (±SEM) motor activity measurements on day 42 by stress treatment group
t2.2 and chlorpyrifos administration

t2.3 Chlorpyrifos[a]	Group[b]	Ambulatory	Non-ambulatory	Total
t2.4 0	1	142.33±18.88	224.00±33.25	366.33±38.91
t2.5	2	123.80±14.63	271.00±25.76	394.80±30.14
t2.6	3	142.67±18.88	248.00±33.25	390.67±38.91
t2.7	4	128.33±18.88	301.00±33.25	429.33±38.91
t2.8 60	1	114.25±16.35	194.50±28.80	308.75±33.69
t2.9	2	111.00±18.88	255.33±33.25	366.33±38.91
t2.10	3	123.25±16.35	251.25±28.80	374.50±33.69
t2.11	4	107.80±14.63	234.60±25.76	342.40±30.14

t2.12 [a]Chlorpyrifos dosage in mg/kg; [b]$n=4$/group, values are numbers of beam breaks in the 30-min test period.

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swimming, running, foot shock and cold of varying durations/exposures (Grauer et al., 2000; Kant et al., 2001; Shaikh and Pope, 2003; Sinton et al., 2000; Song et al., 2002).

Relative to the above, varying results have been found when assessing the ability of stress to enhance permeability of the blood–brain barrier, a not unexpected event given the variety of models. Heat stress (colonic temperature of 40.5 °C for up to 135 min) in mice increased permeability to Evans blue and horseradish peroxidase tracers (Wijman and Shivers, 1993). Thirty minutes of stress enhanced movement of Evans blue from the blood to the brain in rats subject to restraint stress for 30 min (Esposito et al., 2001). Alternatively, Song et al. (2002) found that single restraint episodes of 60 or 90 min or 14 daily repeated ones of 60 min did not alter permeability to horseradish peroxidase in rats.

While much attention has been paid to carbamates, there are few studies of stress-induced alteration of organophosphate esterase inhibition. As noted above, a model of chronic stress (long-term corticosterone in the drinking water) had no effect on delayed neurotoxicity induced by tri-*ortho*-tolyl phosphate, including associated inhibition of neurotoxic esterase (Jortner et al., 2005). Acute stress (60 min of treadmill running) had no effect on the acute toxicity of paraoxon as measured by cholinesterase inhibition (Shaikh and Pope, 2003). Pung et al. (2006) demonstrated that daily restraint or swimming stress exposures over a 28-day period did not alter the inhibitory effect of a single 160 mg/kg dose of chlorpyrifos on brain acetylcholinesterase. Our work expands on these findings. We have shown that extending the stress exposure period to 6 weeks and reducing the dose of chlorpyrifos to 60 mg/kg also failed to elicit a stress-associated alteration of the anticholinesterase effect of the organophosphate. There was an important difference in the interval between stress and chlorpyrifos exposure in these two studies. While Pung et al. (2006) administered the organophosphate insecticide 4 h after stress, we administered chlorpyrifos immediately following a stress exposure. Since we show that stress-induced elevation of plasma corticosterone is transient (Fig. 4), in our dosing schedule chlorpyrifos would have been given when this hormone was at a high blood level since chlorpyrifos is rapidly absorbed (Timchalk et al., 2002). The opportunity for a chlorpyrifos–stress interaction should have been particularly possible in rats exposed to swimming, which had the most profound corticosterone responses. Although stress has profound physiological effects, this study suggests these do not alter the cholinesterase inhibiting effects of organophosphates, at least in situations where stress is induced by restraint and/or swimming and when chlorpyrifos is used as the toxicant.

Specifically, in our experiment, this association of organophosphate exposure and concurrent elevated hormone levels did not lead to subsequent alteration of toxicant-induced inhibition of brain acetylcholinesterase.

Acknowledgment

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